Neuroprotection in Parkinson's Disease:

Controlling Mitochondria-Dependent Apoptosis

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Declaration

I, Chientai Hong confirm that the work presented in this thesis is my own. The help and contribution of others to this thesis is specified in the acknowledgements section. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

ABSTRACT

Objective: To investigate the neuroprotective approach of controlling mitochondria-dependent apoptosis in Parkinson's disease cellular models.

Background: Parkinson's disease is a neurodegenerative disease and there is evidence indicating that mitochondria-dependent apoptosis is related to dopaminergic neuron loss. pUL37x1, an immediate early protein expressed during cytomegalovirus infection, modulates mitochondria-dependent apoptosis by inactivating Bcl-2-associated X protein (Bax), and meclizine is believed to increase glycolysis and hyperpolarize mitochondria, which inhibits apoptosis.

Methods: The neuroprotective and anti-apoptotic effects of pUL37x1 over-expression and meclizine were investigated in SH-SY5Y, a neuroblastoma cell line and primary rat cortical culture cells. Cell death was induced by either staurosporine or 6-hydroxydopamine and measured by lactate dehydrogenase release and propidium iodide binding assay. Apoptotic markers were measured by the release of cytochrome c and the activation of caspase-3. Mitochondrial membrane potential was measured by Tetramethylrhodamine, methyl ester fluorescence obtained by confocal microscope. Extracellular acidification rate (ECAR), a glycolytic activity parameter and oxygen consumption rate (OCR) were measured by XF analyser. Statistics was performed by either ANOVA with Dunnett's post-hoc analysis or two-tailed Student's t-test.

Results: Both pUL37x1 over-expression and meclizine significantly protected against toxin-induced cell death in SH-SY5Y and rat primary cortical culture cells.

Both approaches also down-regulated apoptosis. In terms of meclizine, the protection resulted from glycolysis-related mitochondrial hyperpolarization. Hyperpolarization and protection would decline following glycolytic inhibition. pUL37x1 over-expression prevented apoptotic cell death by two means: Bax-dependent and glycolysis-dependent mechanisms. First, pUL37x1 over-expression led to Bax mitochondria-translocation, and in contrast to control, Bax silencing did not provide more protection on pUL37x1 over-expressing cells. Second, pUL37x1 over-expression increased cellular glycolysis and hyperpolarized mitochondria, and glycolytic inhibitors attenuated the protection, indicating a glycolysis-dependent protective mechanism.

Conclusions: The success of neuroprotection by pUL37x1 and meclizine in Parkinson's disease cellular models not only confirms the significance of controlling mitochondria-dependent apoptosis, but also indicates two novel approaches to neuroprotection.

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1 Introduction

1.1 Parkinson's Disease

1.1.1 Clinical Presentation and Epidemiology of Parkinson's Disease

1.1.1.1 History of Identification of Parkinson's Disease

People have been aware of Parkinson's disease since the times of ancient China and India. Yellow Emperor's Internal Classic, the first Chinese medicine classic written around 425-221 BC, described a Parkinson's disease-like symptom with tremor and stiffness (Zhang et al., 2006). Ancient Indians named the disease 'Kampavata' and treated it with levodopa-containing *Mucuna pruriens* (Manyam, 1990). In the second century, the Greek physician Galen described a disease with rest tremor, postural change and paralysis, which was almost identical with Parkinson's disease (Garcia Ruiz, 2004) and later, in 17th and 18th centuries, some symptoms of Parkinson's disease, such as rest tremor and festination, were noted by doctors. (Goetz, 2011).

Before the middle of 19th century, Parkinson's disease was known as *paralysis agitans* (shaking palsy). It's present name was adopted following the publication of Dr. James Parkinson's report "An essay on the shaking palsy" in 1817 based on his observations of six patients. In this first medical document about Parkinson's disease, he described some key manifestations of the disease, including resting tremor, stooped posture and festinating gait. Dr Charcot, a French neurologist, suggested replacing the term '*paralysis agitans'* by 'Parkinson's disease', in 1872, as there was no marked muscle weakness and that tremor was not an essential

symptom of the disease. Dr Charcot also distinguished between Parkinson's disease and other tremulous disorders, such as multiple sclerosis, and identified Parkinsonism-plus syndromes (Goetz, 2011).

The treatment of Parkinson's disease progressed in line with the increased understanding of the disease. Anticholinergics have been prescribed since the middle of 19th century. The medical treatment of the disease entered a new era in 1960s due to the discovery of levodopa followed by dopamine agonists, amatadine, monoamine oxidase B (MAO-B) inhibitors and catechol-O-methyl transferase (COMT) inhibitors. Alongside this surgical intervention provided another treatment option for Parkinson's disease. Dr Cooper introduced thalamotomy as a treatment of Parkinson's disease in 1950s, but lesionectomy became less popular after the development of levodopa until the emergence of deep brain stimulation in 1990s(Rascol et al., 2011b). However, both medical and surgical treatments were only symptomatic therapies. The concept of a cure for the disease by either neuroprotective treatment or cellular replacement has been investigated for decades but none have, as yet, achieved the goal to cure the disease (Ganz et al., 2011; Stocchi and Olanow, 2013).

1.1.1.2 Motor and Non-motor Symptoms of Parkinson's Disease

The most remarkable clinical manifestations of Parkinson's disease are the motor symptoms, some of which have been described for centuries(Goetz, 2011; Zhang et al., 2006). The cardinal ones are resting tremor, rigidity, bradykinesia, flexion posture of neck, trunk and limbs, loss of postural reflex, and freezing phenomenon(Fahn, 2003). These are essential for a diagnosis of Parkinson's disease.

According to UK Parkinson's disease society brain bank clinical diagnostic criteria, bradykinesia and one of the following three symptoms: muscular rigidity, 4-6Hz rest tremor or postural instability not caused by primary visual, vestibular, cerebellar, or proprioceptive dysfunction are fundamental for diagnosis of Parkinson's disease(Hughes et al., 1992). As well as these well-known symptoms, some additional motor manifestations result from Parkinson's diseases, such as hypomimia, dysarthria, dysphagia, micrographia, scoliosis and camptocormia(Jankovic, 2008).

However, the presenting symptoms of Parkinson's disease are not limited to motor problems. Even in the 19th century, Dr Charcot noticed that dysautonomia and pain accompanied with Parkinson's disease(Goetz, 2011). Nowadays, neurologists recognized five major spectrums of non-motor symptoms in Parkinson's disease, including neuropsyciatric disorders, sleep disorders, autonomic disorders, gastrointestinal disorders and sensory symptoms. Even more important is that some of these non-motor symptoms present years before the clinical motor symptoms. For example, rapid eye movement sleep behaviour disorder, constipation, olfactory deficit and depression have been strongly correlated to the pre-clinical characteristics of Parkinson's disease(Chaudhuri et al., 2006).

1.1.1.3 Epidemiology of Parkinson's Disease and the Impact on Quality of Life

Parkinson's disease affects a great number of people and, in general, the prevalence is 0.3% worldwide(de Lau and Breteler, 2006). It is an age-related

disorder and rarely develops in patients below the age of 50, but the prevalence increases to 1% in people over 60 years old, and rises to 4% in over 80 year olds(de Rijk et al., 1995; Nussbaum and Ellis, 2003). In addition, according to different epidemiological surveys from various countries and races, the disease prevalence seems to be lower in Asian and Black populations compared to White and , is slightly male-predominant, with a male to female ratio of 0.9-2.6.(de Lau and Breteler, 2006).

Parkinson's disease has crucial negative impacts on quality of life. The motor symptoms, non-motor symptoms and medication-related effects all contribute to this. Among all these factors the most significant to quality of life are shuffling, difficulty turning, falls, difficulty in dressing, fatigue, confusion, autonomic disturbance particularly urinary incontinence, unpredictable on/off fluctuations, and sensory symptoms such as pain (Rahman et al., 2008). Parkinson's disease also leads to an economic burden for the patients and society. Average disease related medical costs around £1400 per annum for a newly diagnosed patient(Kaltenboeck et al., 2012), which could result in total costs of up to £62,147 for an advanced patient who spent more than 75% of their time in off-status(Findley et al., 2011).

1.1.2 Pathology of Parkinson's Disease

1.1.2.1 Basal Ganglia Pathology

The hallmark pathological observation of Parkinson's disease is the loss of pigmentation of substantia nigra and locus ceruleus pontis(Lewis, 1971). In the 19th century, James Parkinson postulated that the lesion responsible was located in the

cervical spinal cord or lower brain stem because of preservation of intellectual function(Pearce, 2001). However, the role of midbrain and substantia nigra in Parkinson's disease was recognized in 1893, when a 38-year-old man with left side Parkinsonism signs was shown to have a 2.5cm lesion in the contralateral substantia nigra. In the early 20th century, Tretiakoff, Foix and Nicolesco's works eventually demonstrated the importance of substantia nigra lesion in Parkinson's disease. Tretiakoff's works into the pathology of Parkinson's disease not only identified the loss of pigmented cells in substantia nigra, but also mentioned a cytoplasmic inclusion in the nigral cells(Lewis, 1971). This inclusion body was identical with one that Friederich Lewy had described in 1912 hence these inclusions are now referred to as Lewy bodies in memory of him.

Lewy bodies are nearly always found in the substantia nigral neuromelanin-positive neurons of Parkinson's disease patients, particularly in the posterolateral regions. They are usually eosinophilic, intracytoplasmic, round and 5-25μm in diameter and most consist of a central body surrounded by a pale-staining halo. The ultra-structural appearance of a Lewy body is like a sunflower with a dense central core of circular shaped structures and a rim of radiating filaments (7 to 20 nm in diameter). The larger filaments are at the periphery, which corresponds to the halo (Pearce, 2001; Gibb and Lees, 1988). Nowadays, the presence of Lewy bodies in the substantia nigra is a gold standard for a pathological diagnosis of Parkinson's disease(Hughes et al., 1992).

1.1.2.2 Extra-Basal Ganglia Pathology

Although the core motor symptoms of Parkinson's disease can be explained by the loss of substantia nigra dopaminergic neurons, there are still plenty of non-motor symptoms which do not result from that lesion. Actually, the first place Dr. Lewy identified Lewy bodies was in the dorsal motor nucleus of the vagus, a region within the medulla oblongata, of Parkinson's disease patients. Since then, Lewy bodies have been found in the both peripheral and central nervous system of Parkinson's disease patients. In peripheral nerves, Lewy body pathology has been found in enteric, sympathetic and parasympathetic neurons, which accounts for certain non-motor symptoms such as constipation, orthostatic hypotension and excessive sweating. In the central nervous system, Lewy bodies are not only found in the dorsal motor nucleus of vagus, but also in the medullary reticular formation, the raphe nuclei, the locus ceruleus, the pedunculopontine nuclei and the ventral tegmental area, suggesting Lewy bodies are widespread in the central nervous system of Parkinson's disease patients(Surmeier and Sulzer, 2013). These pathological findings are also able to explain some non-motor symptoms like dementia, depression and rapid eye movement sleep behaviour disorder.

1.1.2.3 The Correlation Between Pathology with the Severity of Disease

Braak and his colleagues were the first group to propose that the distribution of Lewy bodies correlated to the severity of disease(Braak et al., 2003a). They studied 110 autopsy cases presented with Lewy body pathology of which only 41 had a clinical diagnosis of Parkinson's disease. Considering the pattern of neuronal inclusion and the severity of disease, they postulated that the progression of Lewy

body pathology starts from medulla oblongata. The pons and midbrain areas become involved later while, in the advance stage, Lewy bodies can be found in the neocortex, including premotor and primary motor cortex. This finding has led to the hypothesis that the pathogenic process of Parkinson's disease can spread trans-synaptically in the central nervous system(Braak et al., 2003b). Some researchers further suggested that certain components within the Lewy body may have prion-like processes in order to progress the inclusions from caudal to rostral(Visanji et al., 2013).

1.2 Pathogenesis of Parkinson's Disease

The pathogenesis of Parkinson's disease is multi-factorial. Although some genetic mutations have been identified that cause hereditary Parkinson's disease, about 90% patients have idiopathic Parkinson's disease(Trimmer and Bennett, Jr., 2009). In the idiopathic group, the aetiology and pathogenesis of disease are still a puzzle and α-synuclein aggregation, mitochondrial dysfunction, excessive oxidative stress and inflammation are all suspected to play a role. Likewise, in hereditary Parkinson's disease, generally classified as either autosomal dominant or autosomal recessive, the disease is associated with α-synuclein aggregation, mitochondrial dysfunction or excessive oxidative stress depending on the loss/gain function of mutant gene.

1.2.1 Pathogenesis in Idiopathic Parkinson's Disease

1.2.1.1 α-synuclein aggregation

The most striking pathological finding of Parkinson's disease is the presence of Lewy bodies and α-synuclein, an 140 amino-acid-long protein, is the main component of these bodies. α-Synuclein belongs to the synuclein family which also includes β-synuclein and γ-synuclein(Breydo et al., 2012). It is predominantly expressed in the brain at the presynaptic terminals and its expression is induced during neuronal development, following determination of neuronal phenotype and establishment of synaptic connections. Based on this specific localization and regulation, it is thought that the physiological role of α-synuclein relates to membrane-associated processes at the presynaptic level. The main function of α-synuclein is thought to be control of neurotransmitter release, providing a subtle break for neurotransmitter release under circumstances of repeated firing, modulating vesicular biogenesis and controlling compartmentalization(Stefanis, 2012). In addition, α-synuclein shares 40% amino acid homology in its amino-terminal portion with molecular chaperone 14-3-3 and binds to many proteins that 14-3-3 binds to as well. With both structural and functional homology with 14-3-3, α-synuclein is suspected to serve as a protein chaperone in physiological conditions(Recchia et al., 2004).

α-Synuclein is natively unfolded. The highly conserved amino-terminal domain of α-synuclein can shift to an α–helical conformation when it binds to phospholipids, which then causes aggregation to form oligomers. Eventually, it forms β-sheet-rich amyloid fibrils. Lipidic environments and some post-translational modifications,

such as phosphorylation at Ser-129 and nitration on Tyr-125, -133, and -136 promote formation of α-synuclein filaments and oligomers(Recchia et al., 2004; Hansen and Li, 2012; McLean and Hyman, 2002; Takahashi et al., 2002). In addition, the aggregation of α-synuclein is also modulated by various factors, including macromolecular crowding, pesticide exposure and interactions with manganese, iron, and aluminium(Breydo et al., 2012).

It is still unclear how α-synuclein causes toxicity. Multiple pathways may be involved, such as blocked endoplasmic reticulum-golgi transport leading to endoplasmic reticulum stress and golgi fragmentation, decreased synaptic vesicle release, impaired energy production and apoptosis induction in mitochondria, accumulation of chaperone-mediated autophagy substrates and proteasomal impairment(Cookson, 2009). Although β-sheet fibrillar α-synuclein is the main component of Lewy bodies, it is still debatable whether the insoluble form is toxic or not(Goldberg and Lansbury, Jr., 2000). Plenty of evidence from post-mortem studies and animal models reveals that the presence of Lewy bodies does not always mediate neuronal loss, indicating that Lewy bodies are not necessarily responsible for all cell death. However, there are increasing data suggesting that α-synuclein oligomers contribute to toxicity. Some studies found that higher oligomer levels were associated with the disease, and others have demonstrated that designed mutant α-synuclein which fails to form β-sheet fibril still induces significant neuronal loss in experimental models(Kalia et al., 2013).

1.2.1.2 Oxidative Stress

Oxidative stress is a state of imbalance between the generation of reactive oxygen species (ROS) with the ability of a biological system to detoxify the reactive intermediates. The majority of ROS are superoxide anion radicals (O_2^-), hydroxyl radicals (•OH) and hydrogen peroxide (H_2O_2)(Dias et al., 2013). Mitochondrial complexes I and III of the electron transport chain are the main sources of superoxide while peroxisomes can reduce them to hydrogen peroxide and convert this to water(D'Autreaux and Toledano, 2007). In the physiological condition, ROS also serve as a signalling molecule so the level of ROS is tightly controlled by adaptive responses such as defensive enzymes, molecular chaperones and antioxidant molecules. However, if production overwhelms clearance, the excessive levels of ROS induces oxidative stress which causes peroxidation of cellular membrane lipids, protein oxidation and fragmentation, and the oxidation of DNA and RNA(Dias et al., 2013).

There are numerous studies concerning oxidative stress and subsequent oxidative damage in the brains of Parkinson's disease. Lipid peroxidation and DNA damage increases in the nigrastriatum of Parkinson's disease (RajaSankar et al., 2009; Dexter et al., 1994) and markers of protein oxidative damage, such as carbonyl modifications of soluble proteins, and markers of lipid peroxidation, including malondialdehyde, 4-hydroxy-2,3-nonenal, and thiobarbituric acid reactive substance are elevated in the substantia nigra of Parkinson's disease(Yoritaka et al., 1996; Dexter et al., 1989; Floor and Wetzel, 1998). In contrast, the level of

glutathione, a natural antioxidant, was lower in Parkinson's disease, which also indicated the over-production of ROS (Pearce et al., 1997; Sofic et al., 1992).

There are several reasons as to why Parkinson's disease is closely associated with oxidative stress. First of all, dopamine tends to auto-oxidate in the dopaminergic neurons inducing a chronic oxidative stress. Dopamine can be metabolized by monoamine oxidase but may also directly react with oxygen and generate quinones and free radicals(Graham, 1978). In addition, increased iron levels in the substantia nigra leads to the reduction of hydrogen peroxide to produce the highly reactive $^{\bullet}OH$ (Jenner, 2003). Mitochondrial dysfunction in the brains of Parkinson's disease is another crucial factor to support the oxidative stress hypothesis. Decreased complex I activity, which is found in the substantia nigra of Parkinson's disease, results in the generation of ROS(Schapira et al., 1989). Complex I inhibitors, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine(MPTP), also cause Parkinson's disease through impaired mitochondrial function and subsequent oxidative stress(Hasegawa et al., 1990).

1.2.1.3 Inflammation and Lysosomal Dysfunction

Inflammation is recognized as a possible pathogenic factor of Parkinson's disease based on some vital epidemiological findings. Patients using non-steroidal anti-inflammatory drugs had lower risk of Parkinson's disease and there was a consistent association between the human leukocyte antigen locus and Parkinson's disease risk from a meta-analyses of genome-wide association studies(Foltynie and Kahan, 2013). Post-mortem studies have also revealed the presence of activated microglia in the substantia nigra of Parkinson's disease (McGeer et al., 1988). It has

also been postulated that excessive activation of microglia leads to over-production of cytokines and other inflammatory mediators, as well as ROS(Tansey and Goldberg, 2010), but activation of inflammation could only trigger neurodegeneration in the presence of α-synuclein. This suggests a link between protein aggregation and inflammation in the pathogenesis of Parkinson's disease (Gao et al., 2008).

The relationship between lysosomal dysfunction and Parkinson's disease emerged after the discovery that glucocerebrosidase (GBA) gene mutation accounts for 4-7% of idiopathic Parkinson's disease(Murphy and Halliday, 2014). The GBA gene encodes the glucocerebrosidase enzyme which is involved in lysosomal function. Post-mortem data revealed that glucocerebrosidase is reduced not only in the substantia nigra of patients with heterozygous GBA mutations, but also in the substantia nigra of sporadic Parkinson's disease patients (Gegg et al., 2012).

1.2.2 Pathogenesis in Hereditary Parkinson's Disease

A genetic aetiology for Parkinson's disease has begun to emerge in the past 15 years. Although hereditary Parkinson's disease accounts for less than 10% of Parkinson's disease patients, understanding the pathogenesis from these hereditary cases can enrich our knowledge about disease mechanism and also help to model the disease experimentally. At present, there are more than 10 gene mutations which have been identified as the loci of hereditary Parkinson's disease(Klein and Westenberger, 2012). Two autosomal dominant hereditary genes, SNCA and LRRK2, and three autosomal recessive ones, Parkin, Pink1 and DJ-1 are discussed below.

1.2.2.1 Autosomal Dominant

The first genetic mutation associated to hereditary Parkinson's disease was found in the α-synuclein (SNCA) gene locus. Clinically, the onset of disease is in the 4th or 5th decade and the presentations are similar to idiopathic Parkinson's disease. However, the progress of disease is more rapid and associated with the early development of dementia. Pathologically, Lewy bodies are widespread in the brain of SNCA mutation patient, including in the limbic and neocortal regions(Polymeropoulos et al., 1996) .

SNCA mutations cause the increased oligomerization of α-synuclein in several ways. A53T and E46K mutations speed up oligomerization of α-synuclein, whereas A30P speeds up fragmentation of the fibrils, which then accelerates the seeding process for forming new fibrils (Hansen and Li, 2012; Polymeropoulos et al., 1996). Also, duplication or triplication of SNCA gene leads to a corresponding 50% or 100% increase in the level of α-synuclein mRNA and protein(Venda et al., 2010). Increasing oligomerization of α-synuclein from genetic mutations induces Parkinson's disease identical to the idiopathic form, which had been described in previous section 1.2.1.1.

Mutations in the leucine-rich repeat kinase 2 (LRRK2) gene are the most frequent known cause of late-onset autosomal dominant and sporadic Parkinson's disease, with a mutation frequency ranging from 0.5% to 40% in different populations(Shulman et al., 2011). Clinical presentations of LRRK2 mutation-related Parkinson's disease include a mid-to-late onset, slow progression, favourable response to levodopa therapy and less chance of dementia. Pathological findings

are those typical of PD in the majority of cases, although there may be some variation with pure nigral degeneration without Lewy bodies have been reported (Giasson et al., 2006) and with tau pathology.

LRRK2 has well-defined GTPase and kinase functions. It also exhibits multiple biological roles in striatal neurotransmission, neuronal arborisation, endocytosis, autophagy and immunity(Trinh and Farrer, 2013), but it is still uncertain how mutations of LRRK2 lead to Parkinson's disease. Researchers have postulated that gain of function caused by LRRK2 mutation is responsible for the neuronal loss in Parkinson's disease. The toxicity of LRRK2 is closely associated with GTPase activity, defects in endocytic vesicular trafficking and autophagy (Nikonova et al., 2012).

1.2.2.2 Autosomal Recessive

Parkin mutations are the most common cause of recessive hereditary Parkinson's disease and account for 10% of early onset patients(Houlden and Singleton, 2012). The clinical phenotype of homozygous or compound PARK2 mutation cases is slow disease progression, levodopa-responsiveness and late motor complications (Lohmann et al., 2003). The pathological findings of Parkin mutation is very unlike the majority of Parkinson's disease, and includes severe neuronal loss and gliosis without lewy bodies in the substantia nigra. These pathological findings echo the previously mentioned concept that Lewy body formation is not always necessary for the pathogenesis of Parkinson's disease.

Parkin is an E3 ubiquitin protein ligase. Similar to other RING finger containing proteins, it targets misfolded proteins to the ubiquitin proteasome pathway for

degradation and loss of E3 ligase function is responsible for the pathogenesis of disease (Thomas and Beal, 2007). Under physiological conditions, Parkin has an important influence on mitochondria. It modulates mitochondrial biogenesis through Peroxisome Proliferator-Activated Receptor Gamma Coactivator-1-α (PGC-1α) (Shin et al., 2011) and alters the balance of mitochondrial fusion/fission by ubiquitination of mitofusin 1&2 (Gegg et al., 2010). Parkin also plays an important role in mitophagy in conjugation with PINK1(Deas et al., 2011).

It is rare that Parkinson's disease results from mutations in PINK1, which only accounts for up to 1% to 8% of the cases with early onset(Singleton et al., 2013). PINK1 is a mitochondria-associated protein kinase and mutations result in loss of the kinase function. Clinically, patients have a later age of onset compared with Parkin mutations, usually presenting in their 5^{th} or 6^{th} decade but slow progression and levodopa-responsiveness similar to Parkinson's disease associated with Parkin mutations(Houlden and Singleton, 2012). At present, the only autopsy from PINK1 mutation associated Parkinson's disease revealed the presence of Lewy bodies (Samaranch et al., 2010).

Both Parkin and PINK1 are essential for mitophagy and PINK1 works upstream to Parkin(Deas et al., 2011). PINK1 detects dysfunctional mitochondria and accumulates on the mitochondrial outer membrane. Mitochondria-localization of PINK1 recruits Parkin to the damaged mitochondria, and subsequently ubiquitination of mitochondrial outer membrane by Parkin. Eventually, the PINK1/Parkin machinery triggers mitophagy for the clearance of damaged mitochondria. Mutations in either PINK1 or Parkin impair the pathway and lead to

accumulation of dysfunctional mitochondria, which triggers the further neuronal loss by oxidative stress and apoptosis(Grenier et al., 2013).

DJ-1 mutations in Parkinson's disease are rare. The clinical presentation of psychological and behavioural disturbances, amyotrophy and cognitive impairment help to distinguish it from other early onset Parkinson's disease(Trinh and Farrer, 2013). It is believed that DJ-I is an anti-oxidant protein which translocates to mitochondria in response to oxidative stress. To date, there is no pathological report from DJ-1 mutations associated Parkinson's disease(Klein and Westenberger, 2012).

1.2.3 The Vulnerability of Dopaminergic Neurons in Parkinson's Disease

Substantia nigral dopaminergic neurons are the most vulnerable population of neurons in Parkinson's disease. Most of the motor symptoms results from the loss of these neurons. However, current pathological findings reveal that lewy body inclusions are not exclusive to this specific area. Therefore, there must be other explanations for the relatively specific degeneration of midbrain dopaminergic neurons.

The aforementioned auto-oxidation of dopamine is a significant source of oxidative stress in the dopaminergic neurons, which could explain their vulnerability. Also, the morphology of the substantia nigral dopaminergic neurons is distinct from neurons elsewhere. These neurons have long, thin, mostly unmyelinated axons and up to 150,000 presynaptic terminals per neuron (Braak et al., 2006; Sulzer, 2007). Mitochondria present in these nigral dopaminergic neurons

are burdened with the high energy demand required to support synaptic activity, compensate for the potential risk of depolarization in the unmyelinated membrane, and sustain axonal transport over long distances(Fujita et al., 2013). Impaired energy production and failure of mitochondrial axonal transport have been shown to induce parkinsonism and the loss of dopaminergic neurons, supporting a role for this energy-failure concept in Parkinson's disease(Schapira, 2008).

A unique characteristic of midbrain dopaminergic cells is the intrinsic pacemaking activity, which is believed to be important in maintaining basal dopamine levels in the striatum. This distinct electrophysiological feature relies on the influx of calcium through L-type Ca^{2+} channels and leads to increase cytosolic calcium(Gonzalez-Hernandez et al., 2010). The excessive episodic calcium needs to be pumped out, which requires adenosine-5'-triphosphate (ATP); failure to meet the demand for ATP or the sustained opening of the calcium channels leads to undesirable increases in cellular calcium and further endoplasmic reticular stress(Paschen and Mengesdorf, 2005). In addition, increased ATP generation inevitably contributes to increased ROS and oxidative stress(Chan et al., 2009). Excessive intracellular calcium, endoplasmic reticular stress and oxidative stress are all notorious for inducing apoptotic cell death and contributing to the vulnerability of nigral dopaminergic neurons.

1.3 The Role of Mitochondria in Parkinson's Disease

1.3.1 The Physiological Characteristics of Mitochondria

Mitochondria are organelles composed of separate and functionally distinct outer and inner membranes that encapsulate the intermembrane space and matrix compartments. They also contain a circular mitochondrial DNA that encodes 13 mitochondrial proteins. Physiologically, mitochondria play an important role in controlling energy supply, calcium homeostasis and apoptosis. The balance of mitochondrial biogenesis and mitophagy regulates the mitochondrial content of the cell and is also responsible for the quality control of mitochondria. As mitochondria are involved in many physiological pathway, it is no surprise that mitochondrial dysfunction leads to several human diseases.

1.3.1.1 Electron Transport Chain and Reactive Oxygen Species

Aerobic glucose oxidation begins with glycolysis in the cytoplasm and the end-product is pyruvate. The pyruvates are then transported into the mitochondrial matrix, followed by conversion to acetyl-CoA and eventual entry into the tricarboxylic acid cycle where they are oxidized to CO_2 and water. The purpose of glucose oxidation is ATP generation and it is completed by the electrons donated from NADH and succinate enter into electron transport chain. The electron transport chain links with the phosphorylation of ADP (ATP synthesis) by a protonmotive force across the mitochondrial inner membrane, generated by its hydrogen pump effect (Nicholls et al., 2002).

The electron transport chain is composed of four major protein complexes (I–IV). Complex I (NADH-Coenzyme Q oxidoreductase) and complex II (succinate-

coenzyme Q oxidoreductase) pass electrons down to an electron transporter, ubiquinone (coenzyme Q). Then the electrons pass down through coenzyme Q-cytochrome c oxidoreductase (complex III), to another carrier, cytochrome c. In the final step, electrons carried by cytochrome c are transferred to cytochrome c oxidase (complex IV) and reduced oxygen to water(Venditti et al., 2013). During this electron transport, protons are pumped out from the mitochondrial matrix to the intermembrane space by complex I, III and IV in order to generate the protonmotive force. Eventually, the protons re-enter the matrix through complex V (ATP synthase) and release energy generated by the electrochemical gradient. ATP synthase utilizes this energy to phosphorylate ADP to ATP (Walker et al., 1995).

ROS are a by-product of the electron transport chain and the electron leak in complex I and III is mainly responsible for the generation of the superoxide species. Electrons carried by NADH are transported to the flavin mononucleotide (I_F) site in complex I, where they normally pass down a chain of Fe–S centres to the ubiquinone-binding site (I_Q). At both the I_F and I_Q sites, these electrons react with oxygen, forming superoxide ($O_2^{\bullet -}$) within the matrix. In complex III, QH_2 binds to the Q_O site, where the electrons can bypass the ordinary route in the Q-cycle and react directly with oxygen to form superoxide that is released to either side of the mitochondrial inner membrane(Jastroch et al., 2010).

1.3.1.2 Calcium Homeostasis

Mitochondrial Ca^{2+} transport was discovered in the early 1960s. Ca^{2+} uptake is driven by the negative mitochondrial membrane potential and consumes the potential. Ca^{2+} enters mitochondria through Ca^{2+} uniporter, a highly selective Ca^{2+}

channel which is sensitive to ruthenium red(Brookes et al., 2004). Ca^{2+} accumulation in the mitochondria is counteracted by mitochondrial Na^{+}/ Ca^{2+} exchangers and mitochondrial H^{+}/Ca^{2+} exchangers, which are insensitive to ruthenium red but inhibited by benzothiazepine analogues(Rizzuto et al., 2012).

Mitochondria and the endoplasmic reticulum work closely to stabilise intracellular calcium homeostasis. When excitatory stimulation induces the influx of Ca^{2+}, leading to the increase in intracellular calcium, endoplasmic reticulum releases Ca^{2+} from the inositol‑1,4,5‑trisphosphate and ryanodine receptors. At this moment, mitochondria serve as a calcium buffer to modulate cytoplasmic calcium concentration. Increased cytoplasmic calcium causes a microdomain of high calcium at the mouth of the mitochondrial Ca^{2+} uniporter channels and allows rapid Ca^{2+} uptake. This Ca^{2+} is not only stored in the mitochondria matrix but also boosts certain mitochondrial metabolism processes(Brini, 2003).

1.3.1.3 Apoptosis

Apoptosis is a process mediating programmed cell death. It is characterised into two pathways: intrinsic and extrinsic. The extrinsic pathway is initiated by stimulation of the surface receptors to extracellular tumor necrosis factor (TNF) or *Fas*. On the other hand, the intrinsic apoptosis pathway is triggered by intracellular stress and mitochondria play an important role in its mediation. This intrinsic cell programmed-death starts with the mitochondrial membrane permeabilization (MMP) regulated by the Bcl-2 (B-cell lymphoma-2) family of proteins. The MMP allows the release of some apoptotic activators, normally sequestered in the mitochondrial intermembrane space, into cytoplasm, inducing full-blown apoptotic

cell death. These activators include cytochrome c, Smac/DIABLO and apoptosis-inducing factor (AIF). As well as being a key component of electron transport chain during oxidative phosphorylation (OXPHOS), cytochrome c plays an important role in caspase activation following MMP. In the cytoplasm, cytochrome c binds to apoptotic protease-activating factor 1(APAF1), and induces oligomerization of APAF1. The conformational change leads to the formation of apoptosome, which further recruits and activates the caspase initiator. Smac/DIABLO binds to the X-linked inhibitor of apoptosis protein (XIAP). The original function of XIAP is inhibition of caspase activity by directly binding to active caspases, such as caspase 9, caspase 3 and caspase 7. Smac/DIABLO directly antagonises this caspase inhibition ability of XIAP. Although the physiological role of AIF in apoptosis is not clear, it has been shown to trigger a caspase-independent cell death.(Tait and Green, 2010; Zamzami and Kroemer, 2001)

1.3.1.4 Mitochondrial Membrane Potential

Mitochondrial membrane potential is the electric gradient across the mitochondrial inner membrane. It is generated by the pumping out of protons during the mitochondrial electron transport chain. Both the electron and pH gradients across the membrane are components of the protonmotive force for the ATP synthesis although the former is the main energy source (Skarka and Ostadal, 2002). Mitochondrial membrane potential is highly negative, approximately -180 mV and the maintenance of it is vital for several mitochondrial functions, such as ATP synthesis, calcium buffering and apoptosis(Nicholls, 2004).

1.3.1.5 Mitochondrial Quality Control

Mitochondria are essential for many vital cell functions. However, the energy-generating organelle is the source of ROS and can be damaged by its own products. The mitochondrial quality control system is crucial as it prevents damaged mitochondria from triggering the apoptotic cell death, especially in the post-mitotic cells, such as neurons(de Castro et al., 2011).

Mitochondria are dynamic organelles and morphological change, regulated by mitochondrial fusion and fission, has a very close relationship with quality control. Mitochondrial fusion allows the repair of unhealthy organelle by healthy ones. In contrast, by means of fission, the damaged part can be separated from the normal part. Defective mitochondria can either be repaired through molecular quality control pathways, or selectively removed through autophagy using a mechanism that involves the PINK1-dependent recruitment of the ubiquitin ligase Parkin. Accumulation of malfunctioning mitochondria activates the apoptotic pathway and leads to cell death (Fischer et al., 2012; McCoy and Cookson, 2012).

1.3.2 Mitochondrial Dysfunction in Parkinson's Disease

1.3.2.1 Idiopathic and Toxin-induced Parkinson's Disease

The relationship between mitochondrial dysfunction and Parkinson's disease was not identified until the 1980s. MPTP is a by-product of the synthesis of a meperidine analogue. It crosses the blood–brain barrier and is oxidized to MPP^+ by MAO-B in glial cells. MPP^+ can be taken up into dopaminergic neurons via the dopamine transporter and become concentrated in mitochondria. MPP^+ inhibits complex I of the electron transport chain and causes dopaminergic neuronal cell

death due to activation of apoptosis(Winklhofer and Haass, 2010). The pathological presentations from the brains of MPTP-induced parkinsonism, however, are not totally identical to idiopathic Parkinson's disease as it shows dopaminergic neuronal loss in substantia nigra without Lewy body formation(Davis et al., 1979). MPTP is widely used as an animal model of Parkinson's disease to reflect dopamine cell loss. In non-human primates, MPTP administration induces parkinsonism symptoms which are similar to the human disease and which are responsive to levodopa and causes the key pathological finding of the loss of dopaminergic neurons in substantia nigra (Jenner, 2009; Langston et al., 1999). Another mitochondrial toxin, rotenone, is also a complex I inhibitor and causes dopaminergic neuronal loss in substantia nigra has been associated with Parkinson's disease(Martinez and Greenamyre, 2012)

In terms of idiopathic Parkinson's disease, the association between mitochondrial dysfunction and loss of dopaminergic neurons in substantia nigra was identified soon after the awareness of MPTP-induced parkinsonism. About a 30% reduction of complex I activity has been reported in the substantia nigra and frontal cortex of Parkinson's disease patients at autopsy (Schapira et al., 1989). Moreover, the reduced complex I activity is not selective to the brain but is also found in platelets from 25% of Parkinson's disease patients, which suggests this reduction may result from genetic or environmental factors(Parker, Jr. et al., 2008; Schapira et al., 1990; Schapira, 2008). In addition, post-mortem studies have shown increases in mitochondrial DNA deletions in the dopaminergic neurons of the substantia nigra from parkinsonian patients(Bender et al., 2006).

The notion of mitochondrial dysfunction in Parkinson's disease was applied to experimental models to identify the mechanisms of dopaminergic neuronal loss. Cybrid cells, containing mitochondria from patients' platelets, demonstrated increased levels of oxidative stress and decreased basal mitochondrial complex I activity with concomitant enhanced susceptibility to cell death by complex I inhibition (Swerdlow et al., 1996). The MPTP/MPP^+ model clearly demonstrated that mitochondrial dysfunction contributed to neuronal death and the pathogenesis of Parkinson's disease in several areas. First, MPP^+ inhibits mitochondrial complex I, which causes the decrease of endogenous antioxidants and the generation of oxidative stress from complexes I and III. Oxidative stress triggers the apoptotic signal, inducing MMP and release of further apoptotic factors such as cytochrome c. Second, inhibition of mitochondrial complex I decreases ATP levels and leads to a bioenergetic crisis, compromising the function of the ubiquitin proteasome system resulting in cell death(Martinez and Greenamyre, 2012). Last but not the least, MPTP/MPP^+ depletes the mitochondrial pool of active glycogen synthase kinase -3β, while simultaneously increasing the cytosolic counterpart, leading to depolarization of the mitochondrial membrane and subsequent caspase activation and cell death(Petit-Paitel et al., 2009).

1.3.2.2 Genetic Parkinson's Disease

PINK1 and parkin are the best genetic models to demonstrate the linkage between mitochondrial dysfunction and Parkinson's disease. As mentioned in the previous section, mutations in either of them causes the impairment of the clearance of damaged mitochondria, leading to accumulations which induce apoptotic cell death.

The majority of evidence that support this concept is obtained from animal models and cellular experiments due to the shortage of post-mortem human data. In the *Drosophila* model, parkin knockout contributed to abnormal mitochondrial morphology, increased apoptotic cell death in the muscle cells and increased oxidative responsive component (Greene et al., 2005; Greene et al., 2003a). *Drosophila* lacking PINK1 exhibit mitochondrial degeneration which leads to apoptosis in flight muscles and mitochondria swollen with reduced ATP levels, mitochondrial DNA, and mitochondrial proteins(Dawson et al., 2010).

Stronger support for this theory is seen in PINK1 and Parkin mutations cell models of Parkinson's disease. Parkin knockout resulted in reduced Bax ubiquitination and more sensitivity to apoptotic cell death in primary cortical neurons (Johnson et al., 2012). Parkin regulates mitochondrial biogenesis through ubiquitination of a parkin interacting substrate (PARIS), and Parkin knockout leads to a reduction of PGC-1α and neurodegeneration (Shin et al., 2011). As well as the aforementioned PINK1/Parkin machinery, which involves mitophagy, the PINK1/Parkin pathway is also in charge of the mitochondrial quality control by controlling mitochondrial dynamics, either by inducing protective mitochondrial hyperfusion or the mitochondrial fission that precedes mitochondrial degradation. Drp1 is a ubiquitinous substrate of Parkin and Parkin mutation or knockout causing the accumulation of Drp1 and further mitochondrial fragmentation (Wang et al., 2011). Another mitochondrial dynamics related protein, Mfn2, is also a substrate of Parkin and the ubiquitination of Mfn2 requires phosphorylation by PINK1(Chen and Dorn, 2013).

The remaining genetic mutations which cause Parkinson's disease also have some relationship with mitochondria. α-Synuclein mutations cause dysmorphic mitochondria with inclusions and damaged mitochondrial DNA in a mouse model. In addition, mutant α-synuclein proteins co-localized with mitochondrial membrane, result in complex I inhibition and increasing mitophagy(Chinta et al., 2010). Another common autosomal dominant genetic mutation, LRRK2, also affects mitochondrial function. G2019S mutation of LRRK2 causes mitochondrial depolarization and decreased cellular ATP levels through mitochondrial uncoupling (Papkovskaia et al., 2012) . The function of DJ-1 is also relevant to mitochondria as loss of DJ-1 results in increased ROS production, reduced mitochondrial membrane potential and MMP(Giaime et al., 2012).

1.4 The Role of Apoptosis in Parkinson's Disease

1.4.1 Apoptosis

Apoptosis, a programmed cell death process, is critically important for the survival of multicellular organisms. It helps to get rid of damaged or infected cells that may interfere with normal function(Portt et al., 2011) and, during development, apoptosis of excess cells contributes to organ and tissue formation(Meier et al., 2000). The word apoptosis originates from Greek meaning "falling off or dropping off", and is used as an analogy of leaves falling off trees or petals dropping off flowers. Apoptotic cells can be recognized by stereotypical morphological changes, such as cell shrinkage, deformation and loss of contact with neighbouring cells, chromatin condensation, blebbing or budding of the plasma membrane, and the presence of apoptotic bodies containing cytosol, condensed chromatin, and

organelles(Saraste and Pulkki, 2000). Necrosis, as opposed to apoptosis, results in a loss of membrane integrity, swelling and rupture of the cells, strong inflammatory response and damage to neighbouring cells(Leist and Jaattela, 2001).

1.4.1.1 Extrinsic Pathway of Apoptosis

The extrinsic pathway of apoptosis is mediated by a sub-group of TNF receptors, a superfamily that includes TNFR, *Fas* and TRAIL. All members of the TNFR family have cysteine rich extracellular subdomains which binds their ligands with specificity and results in the trimerization and activation of the respective death receptor. Activation of these death domain-containing receptors leads to the recruitment and activation of initiator caspases such as caspases 8 and 10 via processes that involve the formation and activation of complexes such as the death inducing signalling complex (DISC). Procaspase-8 molecules at the DISC result in autocatalytic activation and caspase-8 activation. Activated caspase-8 further triggers downstream activation of effector caspases, typically caspase 3. Activated caspase 3 is responsible for the cleavage of a number of so-called death substrates that cause the well-known characteristic hallmarks of apoptotic cell including DNA fragmentation, nuclear fragmentation, membrane blebbing and other morphological and biochemical changes(Portt et al., 2011; Naismith and Sprang, 1998; Scaffidi et al., 1998). As well as acting on downstream effector caspases, activated caspase-8 also initiates mitochondria-dependent apoptosis by cleavage of Bid. The truncated form of Bid then translocates to the mitochondria where it acts in concert with the proapoptotic Bcl-2 family members Bax and Bak to induce the

release of cytochrome c and other mitochondrial proapoptotic factors into the cytosol(Luo et al., 1998).

1.4.1.2 Intrinsic Pathway of Apoptosis

The intrinsic pathway of apoptosis is induced by a variety of stimuli such as DNA damage, oxidative stress, starvation or cytotoxic drugs(Kaufmann and Earnshaw, 2000; Wang, 2001). These stresses lead to the activation of BH3-only members by transcriptional upregulation (Noxa, Puma), subcellular relocalization (Bim, Bmf), dephosphorylation (Bad),or proteolysis (Bid). Activated BH3-only proteins inhibit anti-apoptotic Bcl-2 members. In addition, they may directly induce a conformational change of Bax and Bak which causes these proteins to oligomerize and insert into the mitochondrial membrane resulting in MMP(Wyllie, 2010). This permeabilization causes the release of apoptogenic factors including cytochrome c, endonuclease G and apoptosis Inducing Factor (AIF). Cytochrome c interacts with pro-caspase 9, APAF-1 and dATP to form an apoptosome. Procaspase-9 molecules can bind to the inner “hub” region of the apoptosome and are activated by dimer formation. Activated caspase-9 dimers further proteolytically activate the effector procaspases-3, -6, and -7, which subsequently found the peptide sequence DEVDG (Asp-Glu-Val-Asp-Gly) in the cells and cleave the carboxy side of the second aspartic acid residue (between D and G). The widespread proteo-cleavage effects result in the amplification of the death signal and eventually in the execution of cell death(Earnshaw et al., 1999). As well as cytochrome c, other factors releasing from mitochondria also trigger the further apoptotic pathway. For example, the release of AIF causes a caspase-independent apoptosis.

MMP also causes several metabolic alterations. The permeability created across the membrane leads to dissipation of the mitochondrial membrane potential, resulting in the loss of ATP synthesis and calcium outflow from matrix.Additionally , ROS generation is increased due to dysfunction of respiratory chain, which amplifies the apoptotic cascade(Kroemer et al., 2007).

1.4.1.3 Modulation of Apoptosis

Apoptosis can be modulated by certain endogenous and exogenous factors acting at various points including the initiation of apoptosis, the balance of pro-apoptotic proteins and pro-survival proteins, some pro-survival pathways and autophagy, a cellular self-repair system(Portt et al., 2011).

Pre-conditioning is a situation where cells experience a brief period of sub-lethal stress. Ischemia/reperfusion is the most well-known model to induce pre-conditioning whilst heat and ROS exposure also work(Stetler et al., 2010; Carriere et al., 2009). Based on different time-courses, there are two types of pre-conditioning. Classical pre-conditioning provides protection minutes after exposure to stress. A number of signalling proteins including extracellular signal-regulated kinases and the Akt pathway are responsible for the anti-apoptotic effect. Late onset pre-conditioning provides long-term protection that lasts 24 to 72 hours. This protection requires protein synthesis and involves the increased expression of a number of genes that encode proteins such as heat shock proteins, anti-oxidants, ceramide utilizing enzymes, as well as a number of other anti-apoptotic genes(Portt et al., 2011).

Cancer cells alter the balance between growth and death in order to keep themselves multiplying. It is therefore not surprising that cancer cells utilise several of these factors and pathways to achieve the goal of anti-apoptosis. Either increased Bcl-2 expression or decreased Bax expression has been found in cancer cells which shifts the balance of Bcl-2 family toward survival(Fulda, 2009; Fulda, 2010). Upregulation of other anti-apoptotic factors including a variety of heat shock proteins, chaperones and genes encoding scavengers of ROS have also been noted(Fulda et al., 2010). Cancer cells can also make use of pre-conditioning and have been shown to up-regulate a number of anti-apoptotic genes to resist chemotherapy treatment(Fulda, 2010).

Apoptosis is induced during the period of viral infection to limit viral replication. So some viral proteins have the ability to modulate apoptosis in order to maintain the viral production. These anti-apoptotic viral proteins can either block the extrinsic apoptotic pathway through generating soluble fake TNF receptor, inhibiting caspase-8 activation, or inducing nuclear factor kappa-light-chain-enhancer of activated B cells (NF-Kb) and c-Jun N-terminal kinases (JNK)-dependent anti-apoptotic pathway. To block the intrinsic apoptotic pathway, virus' can modulate apoptosis by inactivating BH3-only proteins, antagonizing the pro-apoptotic Bcl-2 family proteins or inhibiting caspase activation(Benedict et al., 2002).

Autophagy is the process of recycling cellular constituents. It is a critical adaptive response to starvation. In addition, physiologically, low levels of autophagy serves as general housekeeping, getting rid of unwanted or damaged material in the cell. Removing damaged mitochondria and endoplasmic reticulum could save the cell

from excessive endoplasmic reticular stress and avoid the initiation of apoptosis by cytochrome c released from unhealthy mitochondria. Under apoptotic stimuli, upregulation of autophgy increases the expression of some anti-apoptotic genes and interferes with the pro-apoptotic function of activated Bax in order to prevent cell death(Portt et al., 2011).

1.4.2 Apoptosis in Parkinson's Disease

The contribution of apoptosis to the loss of nigrostriatal dopaminergic neurons in Parkinson's disease remained controversial during the 1990s(Mochizuki et al., 1996; Anglade et al., 1997; Kosel et al., 1997; Banati et al., 1998), due to the dynamic and short-lived nature of apoptosis(Elmore, 2007). The number of apoptotic neurons in brains of Parkinson's disease at a given time cannot reflect the whole course of disease. During this time, the use of terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) staining limited the sensitivity of apoptosis detection. However, after the emergence of combined TUNEL staining with fluorescent DNA binding dyes to detect chromatin condensation and DNA cleavage, the role of apoptosis in Parkinson's disease was confirmed(Tatton et al., 1998; Tatton, 2000). Increased levels of apoptotic signal molecules were found in the post-mortem studies. Immunoreactive Bax, increased immunostaining of caspase-3, caspase-8, and caspase-9 and elevated caspase 1 and 3 activities were identified in the substantia nigra of Parkinson's disease patients(Tatton, 2000; Hartmann et al., 2000; Hartmann et al., 2001b; Mogi et al., 2000).

MPTP produced parkinsonism results in apoptosis of nigrostriatal dopaminergic neurons in mouse models(Tatton and Kish, 1997) as some apoptotic markers, such

as the release of mitochondrial cytochrome c and the activation of caspase-9 and caspase-3 are increased in MPTP treated mice. Moreover, MPTP leads to Bax upregulation through activated p53 and the translocation of Bax to the mitochondria, which can be prevented by the genetic ablation of Bax(Perier et al., 2005; Vila et al., 2001). MPTP also activates caspase-8 and the extrinsic apoptotic pathway(Perier et al., 2012).

Considering the genetic models of Parkinson's disease, virtually all types of genetic mutation enhance apoptosis. Apoptotic markers were found in neocortex, brainstem, and spinal cord of A53T α-synuclein mutant mice and over-expression of α-synuclein has been shown to induce apoptosis in dopaminergic neurons through activation of caspase-9 and caspase-3(Martin et al., 2006; Yamada et al., 2004). Parkinson's disease related LRRK2 mutation increases the level of Bim, a pro-apoptotic BH3 only protein and apoptosis in the *Drosophila* model, while in the neuroblastoma cell line, over-expression of mutant LRRK2 activates caspase-3 and induces apoptosis(Iaccarino et al., 2007; Kanao et al., 2010). Flight muscles of *Drosophila* parkin null mutants undergo apoptotic degeneration, while parkin prevents cytochrome c release in an E3 ubiquitin ligase-dependent manner and protects against apoptosis (Greene et al., 2003b; Darios et al., 2003). DJ-1 prevents apoptosis by inhibiting p53 transcriptional activity and Parkinson's disease related mutation or knockdown of DJ-1 results in hypersensitivity to apoptosis(Fan et al., 2008; Shinbo et al., 2006).

Over-expression of PINK1 attenuates apoptosis by reducing mitochondrial release of cytochrome c and the subsequent activation of caspases(Wang et al., 2007). On

the other hand, mutation or depletion of PINK1 sensitizes the cell to apoptotic death(Venderova and Park, 2012).The anti-apoptotic effect of PINK1 is related to its phosphorylation of two substrates, TNFR-associated protein 1 (TRAP1) and HtrA2. Phosphorylation of TRAP1 by PINK1 prevents cytochrome c release and apoptotic cell death induced by hydrogen peroxide. Phosphorylation of HtrA2 protects mouse embryonic fibroblasts against 6-hydroxydopamine, rotenone, or stress-induced apoptosis(Perier et al., 2012).

1.5 Mitochondria-dependent Apoptosis as a Target of Neuroprotection in Parkinson's Disease

1.5.1 Mitochondria-dependent Apoptosis

Mitochondria are essential in the apoptotic pathway. As mentioned in the previous sections, the intrinsic pathway of apoptosis is not only initiated from mitochondria, but can also be triggered by part of the extrinsic apoptotic pathway through the activation of caspase-8. The term 'mitochondria-dependent apoptosis' refers to the signal pathway of apoptosis that relies on mitochondria involvement. Considering all the apoptotic events which happen in the mitochondria, MMP is the most critical point in mitochondria-dependent apoptosis because it is the point of no return in the majority of circumstances. Once MMP takes place, the apoptotic cascade is triggered by the release of mitochondrial apoptotic factors.

MMP is tightly controlled and the key modulating factors are the Bcl-2 family of proteins. The Bcl-2 family can be divided to three groups. The first group of proteins are anti-apoptotic and include Bcl-2, Bcl-xL, Bcl-w, A1 and Mcl-1. In general, these anti-apoptotic Bcl-2 proteins attach to the mitochondrial outer membrane, inhibit

MMP, bind to and sequester pro-apoptotic Bcl-2 proteins. The second group of proteins are pro-apoptotic and include Bax and Bak. Following an apoptotic stimulus, Bax and Bak attach to the mitochondrial outer membrane and undergo conformational changes that lead to the formation of homo-oligomers, these then form pores in the outer membrane and induce the permeabilization. The last group are the BH3-only proteins, including Bim, Bid and Puma. They do not permeabilize the outer mitochondrial membrane like Bak and Bax, but promote the activation of Bak and Bax through either direct activation of them and/or neutralization of the anti-apoptotic proteins(Parsons and Green, 2010)

Hyperpolarization of mitochondria tends to reduce the permeability while mitochondrial depolarization, caused by Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (CCCP), results in the opposite outcome(Petronilli et al., 1993). Additionally, acidification of the mitochondrial matrix, cyclophilin D and quinones can all lead to less permeability (Bernardi et al., 1999).

MMP could be modulated by BH3 mimetics as well. Antimycin A is a Bcl-2 antagonist, which binds to Bcl-2 in competition with a peptide corresponding to the BH3 domain of Bak and causes collapse of mitochondrial membrane potential and mitochondrial swelling through MMP(Tzung et al., 2001). Some signalling molecules, including the phosphatases and kinases acting on Bcl-2-like proteins, as well as transcription factors, including p53 and TR3/Nur-77/NGFI-B, could trigger MMP and apoptosis. A neutral or acidic environment may also lead to less chance

of MMP in contrast to a maximum permeability at a matrix pH around 7.3(Zamzami and Kroemer, 2001).

However, MMP does not always result in apoptosis. On some occasions, the protective and recovery systems rescue the cell, and these situations are most frequently seen in post-mitotic cells, such as neurons and cardiomyocytes. The insufficient release of cytochrome c from mitochondria may account for this MMP without apoptosis as levels fail to reach the threshold required to trigger caspase activation (Khodjakov et al., 2004). In addition, these cells may have less apoptotic protease-activating factor 1 expression and are less responsive to exogenous cytochrome c-induced apoptosis(Wright et al., 2004; Potts et al., 2005). A high level of glycolysis contributes to the rescue as well as this raises the intracellular levels of glutathione synthase (GSH) though the pentose–phosphate shunt. GSH reduces and inactivates cytochrome c which subsequently inhibits caspase activation(Tait and Green, 2010).

1.5.2 Neuroprotection in Parkinson's Disease

1.5.2.1 Definition of Neuroprotection

Neuroprotection is defined as the prevention of neuronal cell death and maintenance of function without necessarily affecting the underlying biochemical mechanisms involved in pathogenesis. At a clinical level, this means stopping the progress of the disease. Neurorescue can be considered a mechanism where established metabolic abnormalities are reversed and normal neuronal function is

restored. Clinically, this would manifest as an improvement in symptoms as well as a halt in the progress of the disease(Schapira, 1999).

1.5.2.2 History of Anti-apoptotic Strategy of Neuroprotection in Parkinson's Disease

Plenty of anti-apoptotic agents have been proven to produce a neuroprotective effect in cellular and animal models of Parkinson's disease. For example, coenzyme Q10 can inhibit mitochondrial depolarization and prevent apoptosis caused by serum starvation, antimycin A, and ceramide(Papucci et al., 2003), and a novel peptide, H-D-Arg-Dimethyl Tyr-Lys-Phe-NH2 (SS-31) is able to inhibit mitochondrial depolarization, MMP, and the release of cytochrome c triggered by several apoptosis inducers. In addition, treatment with SS-31 has also demonstrated complete protection against MPTP neurotoxicity(Chaturvedi and Beal, 2008). These examples illustrate that apoptosis-targeting strategies are effective for the neuroprotection in both *in vitro* and *in vivo* models of Parkinson's disease.

Unfortunately, two promising anti-apoptotic agents failed to demonstrate the neuroprotective effect in clinical trials. The propargylamine TCH346 is an anti-apoptotic factor that inhibits the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which can initiate apoptosis. The neuroprotective effect of TCH346 was highly potent in both 6-OHDA and MPTP animal models, however, in a double-blind, randomized trial involving 301 patients it failed to show a significant difference in clinical outcome(Olanow et al., 2006). Another anti-apoptotic agent, CEP-1347, an inhibitor of mixed lineage kinases that can activate the c-Jun N-terminal kinase (JNK) pathway involved in cell death, also showed encouraging

results in preclinical studies, but again, the PRECEPT trial, involving 806 patients with early Parkinson's disease, was terminated early when an interim analysis revealed a lack of efficacy in the experimental treatment(2007).

The discrepancy between the promising results in the animal models and the failure of clinical trials raises questions about the application of anti-apoptotic agents for neuroprotection in Parkinson's disease. However, it is worth mentioning some of the difficulties in the clinical trials of Parkinson's disease. Currently, there are no good biomarkers with which to measure disease progression. Clinical motor symptoms are the main parameters used to assess the effectiveness of neuroprotection, but they are not necessarily a good reflection of neuronal viability(Kieburtz and Ravina, 2007). Although most trials enrol patients with early motor symptoms, their brains have already undergone a more than 50% cell loss in the substantia nigra(Cheng et al., 2010) and Lewy bodies are present in the lower brainstem before motor symptoms are evident. This situation results in an unfavourable environment for the neuroprotective agent in clinical trials so considering these issues, the unpromising outcomes of previous trials should not prevent further attempts to utilise anti-apoptotic neuroprotection in Parkinson's disease

1.5.3 Target on Mitochondria-dependent Apoptosis in Parkinson's Disease

1.5.3.1 Mitochondria-Dependent Apoptosis, an Ideal Target for Neuroprotection

Based on the statements in the previous sections, mitochondrial dysfunction, apoptosis and Parkinson's disease have a clear relationship. With the complex connections with each other, it is natural to postulate that modulation to either mitochondrial dysfunction or apoptosis would prevent the loss of dopaminergic neurons in Parkinson's disease brain. However, it is difficult to either prevent or rescue mitochondria from dysfunction in Parkinson's disease because many of the environmental factors which cause mitochondrial dysfunction have not yet been fully identified. Moreover, there is no practical way to amend the genetic mutations which cause mitochondrial dysfunction. In addition, Parkinson's disease is a multi-factorial disease and protein misfolding, neuroinflammation and lysosomal dysfunction are all relevant to the disease. In contrast, the contribution of apoptosis in Parkinson's disease has been widely studied. Generally speaking, all the known pathogenetic pathways of Parkinson's disease trigger neuronal death through apoptosis. Modulating a shared, common pathway of cell death is logical for a multi-factorial disease like Parkinson's disease.

Unlike the lack of good options to restore mitochondrial function in Parkinson's disease, there are plenty of apoptosis inhibitors available. Unfortunately none of them act on a common target point in apoptosis, for example, caspase inhibitors are very potent for inactivating these executors of apoptosis, but have no influence

on caspase-independent apoptosis. Mitochondria are involved in both extrinsic and intrinsic pathways and the accumulation of damaged mitochondria, which is the signature characteristic of Parkinson's disease, can trigger apoptosis, making them a key target for future therapies.

In summary, the trinity that is Parkinson's disease, apoptosis and mitochondria intertwine with each other and blocking mitochondria-dependent apoptosis could be a tailor-made neuroprotection for Parkinson's disease.

1.5.3.2 Maintain Mitochondrial Membrane Potential for Anti-apoptosis

The collapse of mitochondrial membrane potential highly correlates to initiation of apoptosis so it is not surprising that keeping membrane potential in a more negative voltage (hyperpolarization) could be anti-apoptotic and, therefore, neuroprotective. Bonnet *et al* found that mitochondrial hyperpolarization is responsible for the apoptosis resistance in cancer cells(Bonnet et al., 2007) and Heerdt *et al* demonstrated that cancer cells with hyperpolarized mitochondria are more resistant to chemotherapy(Heerdt et al., 2005). In human mesenchymal stem cells, a more negative mitochondrial membrane potential correlated with less apoptosis and cell death(Pietila et al., 2010).

Maintaining mitochondrial membrane potential by hyperpolarization could protect cells against apoptotic stimuli in several ways. Membrane potential is responsible for calcium storage and ATP synthesis. Depolarization during apoptosis causes calcium release and cessation of ATP synthesis, which would amplify the apoptotic stimuli due to increase intracellular calcium and energy depletion(Kroemer et al.,

2007), and depolarization enhances the permeability of the mitochondria membrane, which also facilitates apoptosis. In addition, mitochondrial membrane potential regulates the mitochondrial matrix configuration and normopolarized mitochondrial membrane potential keeps cytochrome c in the cristae and resistant to release by agents that disrupt the mitochondrial outer membrane(Gottlieb et al., 2003).

The potential gradient across the mitochondrial inner membrane is generated by pumping out protons from the electron transport chain and these protons re-enter the matrix through complex V (F_1F_0-ATP synthase) accompanied with ATP synthesis. Either inhibiting complex V by oligomycin or insufficient ADP supply for ATP synthesis interferes with the protons re-entry and leads to mitochondrial hyperpolarization. However, these situations also damage ATP synthesis and are not beneficial. On the other hand, increasing the supply of NADH from the glycolytic pathway boosts the electron transport chain and result in hyperpolarization without damaging ATP synthesis. Glycolysis-generated ATP can also be utilized in complex V, where the role of F_1F_0-ATP synthase changes to consume ATP and pump out protons to increase the potential gradient(Iijima, 2006). In the PINK1 mutant model, myocytes but not neurons are thought to make use of glycolysis-generated ATP to maintain mitochondrial membrane potential and make them more resistant to apoptotic stimuli(Yao et al., 2011).

1.5.3.3 Modulate the Bcl-2 Family Proteins for Anti-apoptosis

Adjusting the balance of pro-apoptotic/anti-apoptotic Bcl-2 family of proteins could modulate apoptosis and provide neuroprotection. Over-expression or

transfection of Bcl-2 , an anti-apoptotic protein, may prevent neuronal loss induced by brain ischemia by inhibiting the release of cytochrome c, activation of caspase-3 and the translocation of AIF from mitochondria to the nucleus(Zhao et al., 2004; Zhao et al., 2003). Modulating Bax also results in neuroprotection in experimental models. Bax channel inhibitors prevented the release of cytochrome c, dissipation of mitochondrial membrane potential, apoptotic cell death *in vitro* and reduced neuronal loss in a global brain ischemia model(Hetz et al., 2005). In a traumatic brain injury model, *bax* gene knockout mice were shown to be more resistant to cell loss, had less apoptotic neurons and lower caspase-3 activity(Gibson et al., 2001; Tehranian et al., 2008). In addition, models with knockout of BH3-only proteins, such as Bid and Bim, also have demonstrated a neuroprotective effect(Galluzzi et al., 2009).

Rasagiline (N-propargyl-1-(R)-aminoindan), a novel and highly potent irreversible MAO-B inhibitor has been studied for neuroprotection in a Parkinson's disease clinical trial(Rascol et al., 2011a). The mechanism of action may include modulating Bcl-2 family proteins. Rasagiline increased the levels of bcl-2 and bcl-x_L mRNA, as well as levels of Bcl-2 protein(Akao et al., 2002; Inaba-Hasegawa et al., 2012). Zhu et al also found that rasagiline protected lactacystin-induced nigrostriatal dopaminergic degeneration and reversed the reduction of Bcl-2 protein level by lactacystin(Zhu et al., 2008).

1.6 Enhance Glycolysis to Maintain Mitochondrial Membrane Potential: Meclizne Model

1.6.1 Glysolysis

1.6.1.1 The Glycolytic Pathway

Glycolysis, a metabolic pathway used by virtually all organisms, provides energy and building blocks for biosynthetic reactions. It is recognized as a most ancient metabolic pathway and occurs in the cytosol(Romano and Conway, 1996). Glycolysis converts glucose to pyruvate and produces 4 ATP's and 2 NADH can work both aerobically and anaerobically due to its oxygen-independent characteristic. However, it also consumes 2 ATP's in the process so the net yield is 2 ATP's and 2 NADH.

Glycolysis begins with the phosphorylation of glucose by hexokinases to form glucose 6-phosphate. The first five steps are the preparatory phase, which consumes energy to convert the glucose into two three-carbon sugar phosphates. The second half is the pay-off phase, which produces ATP and NADH. The end-product of glycolysis, pyruvate, is taken into mitochondria and converted to acetyl-coenzyme A, which is fully oxidized to CO_2 through the tricarboxylic acid cycle if sufficient oxygen is available. However, under hypoxic conditions or following inhibition of mitochondrial respiration, pyruvate is converted into lactate and shunted out of the cell.

1.6.1.2 The Physiological Characteristics of Glycolysis

Glycolysis is an alternative energy resource to OXPHOS, and though it might be seen as bioenergetically inefficient, due to low ATP and NADP yield, because of the fast reaction rate, glycolysis is able to provide sufficient energy to meet cellular demand(Pfeiffer et al., 2001). In addition, the nature of the fast reaction can be utilized by certain tissues in order to rapidly meet energy demand. Myocytes tend to have higher glycolytic ability because of their fast and intensive contraction. The variability of glycolytic activity between different tissues many account for the disparities in resistance to hypoxia or energy deficit. PINK1 KO myocytes make use of higher glycolytic activity to prevent cell death from mitochondrial dysfunction, whereas neurons, that could not boost glycolysis, would die(Yao et al., 2011).

Cancer cells usually have higher glycolytic activity regardless of the oxygen concentration, which is known as the Warburg effect. In these cancer cells, glycolysis is the main energy resource and it works regardless of the aerobic conditions. Shifting the energy supply from mitochondria to glycolysis results in less ROS generation from electron transport chain and more resistance to apoptosis(Wallace, 2005). Also, glycolysis enhances multiplication of cancer cells and the intermediates of glycolysis could also be the building blocks for the daughter cells(Bolanos et al., 2010).

1.6.1.3 The Regulation of Glycolysis

Glycolysis is tightly regulated at several stages. The purpose of this regulation is to meet the demand for ATP cellular energy and factors for synthetic reactions. During glycolysis, glucose is converted to pyruvate and the whole process is made

up of ten steps and each step requires a specific catalysing enzyme. Among these ten steps, only three are almost irreversible and they are the points of glycolysis control.

Phosphofructokinase is the most crucial control point in the glycolytic pathway. The enzymatic activity is tightly regulated by the ATP/AMP ratio: high levels of ATP allosterically inhibits the enzyme while AMP reverses the inhibitory action of ATP. Therefore, glycolysis is stimulated by deficits in energy charge. This key glycolytic enzyme is also modulated by citrate, an early intermediate in the citric acid cycle, however the inhibitory action of citrate is ATP-dependent. A high level of citrate means that biosynthetic precursors are abundant and additional glucose should not be degraded for this purpose(Mor et al., 2011).

Hexokinase, the enzyme responsible for the first step of glycolysis, is also a regulatory point due to its irreversible characteristic. Its product, glucose 6-phosphate, negatively feedbacks the reaction because higher intracellular glucose 6-phosphate indicates an energy-rich status of cell(Wilson, 2003). Pyruvate kinase which controls the outflow of glycolysis is the third irreversible enzyme. The destination of pyruvate, a central metabolic intermediate, is decided by the levels of cellular ATP. High energy charge status results in inhibition of pyruvate kinase and slowing down of glycolysis, or shifting the intermediates to be the build blocks for synthetic reactions(Xiong et al., 2011).

Glucose transporters, which manage the entrance of glucose into the cell, also modulate glycolysis. GLUT1 and GLUT3 are responsible for basal glucose uptake due

to the low K_M value for glucose. They continually transport glucose into cells at an essentially constant rate. GLUT4, with a moderate K_M value of glucose, transports glucose into muscle and fat cells. The abundance of GLUT4 is regulated by the presence of insulin which promotes the uptake of glucose by muscle and fat(Tirone and Brunicardi, 2001).

In experiments, glycolysis can be upregulated by increasing glucose concentration in the feeding medium(Williams et al., 2007), over-expressing certain glycolytic enzymes(Herrero-Mendez et al., 2009) or pre-conditioning with sub-lethal stress, including hypoxia and hydrogen peroxide (Wu and Wei, 2012; Malthankar-Phatak et al., 2008). Mitochondrial respiratory chain inhibition by MPP^+ or rotenone also results in a compensatory glycolysis boost(Campello et al., 2013; Giordano et al., 2012). Cancer cells are known to enhance glycolysis through several mechanisms, including increasing expression of glucose transporters, glycolytic enzymes and stabilizing hypoxia inducible factor 1α (HIF-1α), which also participates in the process of pre-conditioning(Cairns et al., 2011).

1.6.2 Glycolysis in Anti-apoptosis and Neuroprotection

1.6.2.1 Anti-apoptosis

As well as being a significant source of cellular energy, glycolysis also plays an important role in apoptosis. The majority of studies focus on cancer cells because they usually have higher glycolytic activity and are more resistance to apoptosis. Chen *et al* found that overexpression hexokinase 2 in laryngeal squamous cell carcinoma is responsible for the anti-apoptotic activity and proliferation of this

cancer(Chen et al., 2013). Decreased expression of fructose-1,6-bisphosphatase-2, an enzyme that works against glycolysis, also promoted tumour growth while over-expression of it caused apoptosis(Li et al., 2013). Similar effects have been shown in non-cancerous cells, for example, up-regulation of glycolysis in Chinese hamster ovary cells upon *tert*-Butyl Hydroperoxide treatment resulted in less cytochrome c release and less apoptotic cells (Jeong et al., 2004).

1.6.2.2 Neuroprotection

Based on the anti-apoptotic activity, it is reasonable to suggest that control of glycolysis in neurodegenerative models may provide neuroprotection. β-Amyloid is highly associated with the pathogenesis of Alzheimer's disease and Newington *et al* showed that β-amyloid resistant neurons exhibit increased glycolysis due to increased pyruvate dehydrogenase kinase 1 and lactate dehydrogenase A and that inhibition of these two enzymes led to re-sensitization to β-amyloid toxicity. They also demonstrated that over-expression of these glycolytic enzymes in neuronal cells resulted in less ROS generation from mitochondria and the persistence of protection against β-amyloid(Newington et al., 2011; Newington et al., 2012). Elevated glycolysis has also been proven to protect neuronal cells from MPTP, a drug frequently used in Parkinson's disease model. Glycolysis was up-regulated by increasing the glucose concentration in the feeding media. The cells given higher glucose utilized glycolysis to generate more ATP and had less dissipation of mitochondrial membrane potential induced by MPTP. In both cell lines and primary cerebellar granule cells, these effects contributed to neuroprotection against MPTP(Williams et al., 2007; Mazzio and Soliman, 2003; Gonzalez-Polo et al., 2003).

1.6.3 Meclizine, a Newly Identified Glysolysis-Enhancing and Neuroprotective Drug

1.6.3.1 Traditional Pharmacological Mechanisms of Meclizine

Meclizine is a widely-used medicine for antiemetic and motion sickness. Traditionally, it is a H_1 receptor antagonist and classified as the first generation of antihistamine in pharmacology. It is also a weak muscarinic acetylcholine receptor antagonist and can cross the blood-brain barrier. The side effects of meclizine are drowsiness, dry mouth, and tiredness. The use of meclizine is relative safe, even during pregnancy(Larrauri et al., 2014).

1.6.3.2 Enhanced Glysolysis and Neuroprotection by Meclizine

Gohil *et al* first identified the enhanced glycolysis effect of meclizine in 2010(Gohil et al., 2010). They used a nutrient-sensitized screen to discover agents that shift energy metabolism. From 3695 agents tested, 25 clinically used agents exhibited glycolysis-enhanced ability and of these, Meclizine was selected to test further due to its safety profile and ability to penetrate the blood-brain barrier,. Meclizine demonstrated a neuroprotective effect in a mouse stroke model. Pre-treatment with meclizine, but not with other antihistamine and anticholinergic agents, significantly reduced the infarct volume. In a following study, meclizine was shown to have anti-apoptotic activity against serum-withdrawal induced apoptosis in mutant huntingtin expressing mouse striatal neurons. In addition, meclizine displayed a protective effect on neuronal dystrophy and cell death in *C. elegans* and *Drosophila* models of polyQ toxicity, and the application of meclizine leads to a

dose-dependent increase in glycolysis. However, the mechanism by which meclizine increase glycolysis without elevation of HIF-1α is still unknown (Gohil et al., 2011).

1.7 Modulate Mitochondrial Outer Membrane Permeability: Human Cytomegalovirus pUL37x1 Model

1.7.1 Cytomegalovirus Infection and Anti-apoptosis

Apoptosis is a powerful innate defence mechanism against viral infection because viruses need the machinery of host cell for their own replication. Cell debris containing viral proteins can be uptaken by antigen presenting cells and used to prime the adaptive immune response. Thus, many viruses, including human cytomegalovirus (CMV), encode proteins that attack this defence system. These anti-apoptotic proteins target the system in different ways, including blocking the activation of caspase-8 in the extrinsic apoptotic pathway, inhibiting the initiation of intrinsic apoptotic pathway at mitochondria and the activation of the survival pathway. Without these proteins, CMV is not able to resist the apoptosis of infected cells and maintain the life long infection.

1.7.2 Anti-apoptotic Role of pUL37x1

UL37 exon 1 protein (pUL37x1), which is also known as a viral mitochondria-localized inhibitor of apoptosis, is encoded by the immediate early UL37x1 gene. Goldmacher *et al* found that transfection of pUL37x1 into HeLa cells resulted in suppression of *Fas*-mediated apoptosis and the mitochondria-localization of pUL37x1(Goldmacher et al., 1999). Hayajneh *et al* later on identified two domains within the pUL37x1, between amino acids 5 to 34, and 118 to 147 that were

responsible for the anti-apoptotic activity by deletion mutagenesis analysis. The first of which targeted pUL37x1 to mitochondria(Hayajneh et al., 2001).

The detailed anti-apoptotic mechanism of pUL37x1 was revealed by Arnoult *et al*, who demonstrated that pUL37X1 formed a complex with Bax and sequestered Bax at mitochondria. Out of a variety of generated mutant pUL37x1s, only the ones with preserved Bax-binding function prevented Bax-mediated MMP, but did not associate with Bak nor suppress Bak-mediated MMP(Arnoult et al., 2004). Subsequently, Norris and Youle showed that the mitochondrial translocation of Bax was triggered by pUL37x1. They also found that m38.5, a functional ortholog of pUL37x1 from murine CMV, protected Bak-KO but not Bax-KO cells from staurosporine-induced apoptosis. These two studies both indicate that pUL37x1 interacts with Bax and that its anti-apoptotic activity is Bax-dependent(Norris and Youle, 2008). By using nuclear magnetic resonance, Ma *et al* detected the binding site of Bax within pUL37x1, which is opposite to the BH1 and BH2 domains, with its N terminus close to the BH3 domain(Ma et al., 2012).

1.7.3 Other Biological Effects of pUL37x1

pUL37x1 not only affects host apoptosis but also modulates several other cellular functions. Some of these are related to apoptosis while others are not. Expressing pUL37x1 in human fibroblast leads to disruption of the mitochondrial network and interferes with mitochondrial fusion/fission balance. This disruption, which is associated with the anti-apoptosis and mitochondrial localization, was absent when pUL37x1 mutants which lack the anti-apoptotic effect were expressed. The phenomenon of fragmented mitochondria may result from the inactivation of Bax

because Bax is thought to maintain mitochondrial elongation(McCormick et al., 2003).

Because of its mitochondrial localization, it is not surprising that pUL37X1 is believed to affect the mitochondrial respiratory chain and ATP synthesis. Poncet *et al* demonstrated that expression of pUL37x1 reduced the cellular ATP level due to ATP synthes inhibition(Poncet et al., 2006). However, Kaarbø *et al* found that although the increase of respiratory capacity, which is accompanied by increased ROS, after CMV infection is pUL37x1 dependent, there did not appear to be any interaction between pUL37x1 and complex V, the ATP synthase. In addition, pUL37x1 enhanced PGC-1α expression, which promotes both mitochondrial biogenesis and viral replication(Kaarbo et al., 2011).

1.7.4 Utilization pUL37x1 in Neuroprotection, a Novel Approach

The anti-apoptotic activity of pUL37x1 has been demonstrated in several studies mentioned previously. In brief, it can protect against a variety of apoptosis pathways, including *Fas*-mediated apoptosis, caspase-3 independent apoptosis and proteasome-inhibitor-induced apoptosis. In addition, the protection was demonstrated in mouse NIH 3T3 cells, human HeLa cells and human fibroblasts. However, it has not been tested as a neuroprotective agent in neurodegenerative disease. Although one recent article revealed that pUL37x1 did not protect CMV infection-induced cell death in human neural precursor cells(Hildreth et al., 2012), the feasibility of using pUL37$_X$1 to protect neuronal death is still unclear. Certainly, one of the other CMV gene products, β2.7 RNA, can protect rotenone-induced apoptosis and rescue dopaminergic cell death *in vitro* and in animal models of

Parkinson's disease. Therefore, based on its strong Bax-related anti-apoptotic effect(Reeves et al., 2007; Kuan et al., 2012), it is reasonable to suspect that pUL37x1 could be beneficial to dopaminergic neurons in Parkinson's disease, which are highly susceptible to mitochondria-dependent apoptosis.

1.8 Hypothesis and Aims

1.8.1 Hypothesis

Apoptosis contributes to the death of dopaminergic neurons in Parkinson's disease; manipulation of mitochondria to prevent activation of apoptosis represents a possible therapeutic approach to provide neuroprotection. I hypothesise that hyperpolarization of mitochondria by meclizine and inactivation of Bax by over-expressing CMV protein pUL37x1 could prevent apoptosis and be neuroprotecitive in Parkinson's disease.

1.8.2 Aims

1.8.2.1 Meclizine Model

1. To confirm the alteration of glucose metabolism by meclizine in the SH-SY5Y model
2. To evaluate the neuroprotective effect of meclizine in the SH-SY5Y model
3. To identify the neuroprotective mechanism of meclizine and its modulation of the mitochondrial membrane potential
4. To confirm the glycolysis-dependent neuroprotective effect of meclizine
5. To investigate the glycolysis-enhanced mechanisms of meclizine
6. To evaluate the neuroprotective ability of meclizine in primary neurons.

1.8.2.2 pUL37x1 Model

1. To generate stable pUL37x1 expression in SH-SY5Y cells and characterise the expression

2. To evaluate the neuroprotective and anti-apoptotic effects of pUL37x1 in stable expression cells

3. To clarify the Bax-dependent neuroprotective mechanism of pUL37x1

4. To investigate other cellular functional alterations of expression and identify the possible beneficial and deleterious effects.

5. To express pUL37x1 in primary neurons and evaluate the neuroprotective effect.

2 Materials and Methods

2.1 Cell culture

2.1.1 Human Neuroblastoma Cell line (SH-SY5Y)

2.1.1.1 Background Knowledge of SH-SY5Y Cell Line

The SH-SY5Y cell line was established from a bone marrow biopsy of a neuroblastoma patient with sympathetic adrenergic ganglial origin(Biedler et al., 1973). The SH-SY5Y cell line is a homogeneous neuroblast-like cell line and exhibits neuronal marker enzymatic activity, norepinephrine uptake ability, and several neuronal receptors, including opioid, muscarinic, and nerve growth factor receptors. It also presents several characteristics similar to dopaminergic neurons, such as dopamine synthesis, dopamine transporter expression and response to dopamine agonists. These characteristics are the reason why SH-SY5Y cell line has been used as a cellular research model of Parkinson's disease(Xie et al., 2010). However, there are some disadvantages which limit the use of this neuroblastoma cell line in Parkinson's disease research. First, it is a dividing cell line which is contrary to the post-mitotic dopaminergic neurons. Besides, it is more resist to neurotoxins and less sensitive to neuroprotective agents than primary neurons(Datki et al., 2003). However, taking all the pros and cons into consideration, SH-SY5Y still receives broad acceptance as a cellular model of dopaminergic neurons in Parkinson's disease research.

2.1.1.2 Methods of Culturing SH-SY5Y Cell Line

The method of growing SH-SY5Y cells has been described previously (Chau et al., 2009). SH-SY5Y cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium (Life Technologies, Paisley, UK) and Ham's F12 medium (Life Technologies, Paisley, UK), supplemented with 10% fetal bovine serum (Life Technologies, Paisley, UK), 1mM pyruvate (Sigma, St. Louis, US), 0.01 mM nonessential amino acids (Life Technologies, Paisley, UK), 100 U/ml penicillin (Life Technologies, Paisley, UK), and 100 mg/ml streptomycin (Life Technologies, Paisley, UK), at 37°C under humidified 5% CO_2 atmosphere (Hera Cell 240, Thermo Scientific, Essex, UK). In over-expressing cell lines, 400μg/ml geneticin (GE Healthcare, Little Chalfont, UK) were added into the medium for the purpose of selection.

The adherent SH-SY5Y cells were passaged or harvested by trypsinization in versene (Life Technologies, Paisley, UK), containing 0.1 % trypsin enzyme (Life Technologies, Paisley, UK). Culture medium was removed and cells were washed once by versene followed by 1 ml of trypsin solution applied to each 10 cm plate. The detachment of cells was monitored under microscope. Trypsin was neutralised with equal volume of culture medium containing serum and cells were re-plated at 1:4 dilutions. SH-SY5Y cells of passage number between 10~25 were used for the experiments. Cell stocks were frozen slowly to -80 ^{o}C in fetal bovine serum containing 10 % dimethyl sulfoxide (DMSO) and stored in liquid nitrogen.

2.1.2 Primary Rat Cortical Culture

2.1.2.1 Background Knowledge of Primary Rat Cortical Culture

In the previous section, the limitations of using neuroblastoma cell lines has been mentioned. The most recognised disadvantage of using cancer transformed cell lines is their different response to neurotoxins and neuroprotective agents comparing to neurons. Primary neuronal culture offers a solution to this problem. Primary culture is the term given to culture cells isolated from tissue *in vitro* without immortalization. Primary neuronal culture is usually obtained from embryonic materials. At prenatal stages neurons have not yet developed extensive neurites, which causes less damage during the preparation process (Sciarretta and Minichiello, 2010). Primary neuronal cultures can mimic the nature and behaviour of neurons *in vivo* so are a popular model for studying neuroprotection.

2.1.2.2 Methods of Primary Rat Cortical Culture

The method for primary rat cortical culture was adapted from previous well-established protocols(Xu et al., 2012). Generally speaking, E18 primary rat cortical cultures were obtained from pregnant Sprague Dawley rats. The pregnant rats were asphyxiated in CO_2 chamber and the death was confirmed by cervical dislocation. Embryos were taken out from the sacs and decapitated. The embryo heads were soaked in phosphate buffered saline (PBS) and brought to the tissue culture hood. In the hood, the skull and meninges were removed and bilateral frontal lobes were collected. Then the cortexes went through trysinization by versene containing 0.1 % trypsin enzyme at 37°C for 15 minutes, followed by neutralization with fetal bovine serum. In order to dissociate the neurons, the cell suspension was triturated and

then passed through a 40µm cell strainer (BD, New Jersy, US). Neurons were cultured on 25µg/cm^2 poly-D-lysine coated cover slips and maintained by Neurobasal Medium with 0.5 mM of GLUTAMAX I, 2% B27 Supplement, 100 U/ml penicillin and 100 mg/ml streptomycin (all from Life Technologies, Paisley, UK). Culture medium was changed every three days. In some experiments, frozen primary rat cortical cultures were obtained from Life Technologie and maintained under the same conditions as mentioned before.

2.1.2.3 Validation of Primary Rat Cortical Culture

The growth of primary rat cortical cultures was monitored carefully. Photomicrographs taken by light microscope illustrated in Figure 2-1 showed the maturation of cultured neurons. On the second day of culturing, some neurons started neurite outgrowth. Two days later, almost all the cells had produced neurites. At the end of first week, not only had the neurites extended in length but they had also formed networks. Morphologically, more than 90% of cells presented with the neuronal features.

Fig 2-1.

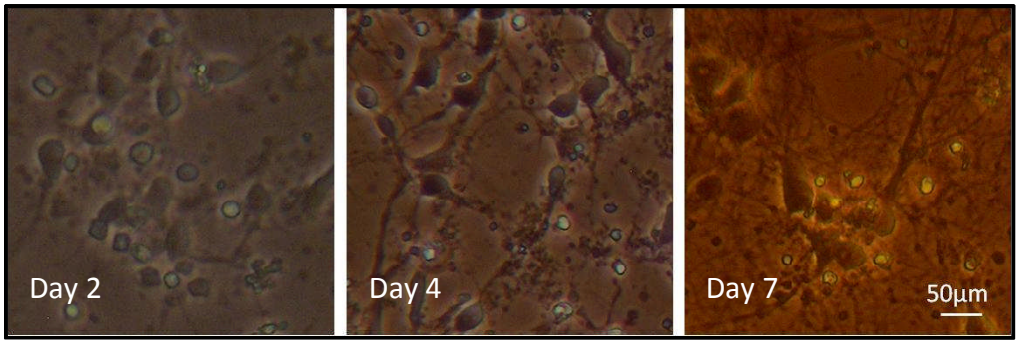

Figure 2-1. Serial pictures of primary rat cortical culture revealed the maturation of neurons. At the 2nd day of culturing, most cells remained round and only some cells demonstrated the outgrowth of neurites. At the 4th day, most cells presented the features of neurons with extension of neurites. One week after the start of culturing, the neurons

developed an extensive neuritic network. Morphologically, most cells were recognized as neurons.

In order to further identify the purity of neurons in the primary rat cortical culture, immunocytochemistry staining with β-III tubulin (neuronal markers) were performed 1 week after the start of culturing. Figure 2-2 showed that the percentage of β-III tubulin positive cells were above 90%.

Fig 2-2

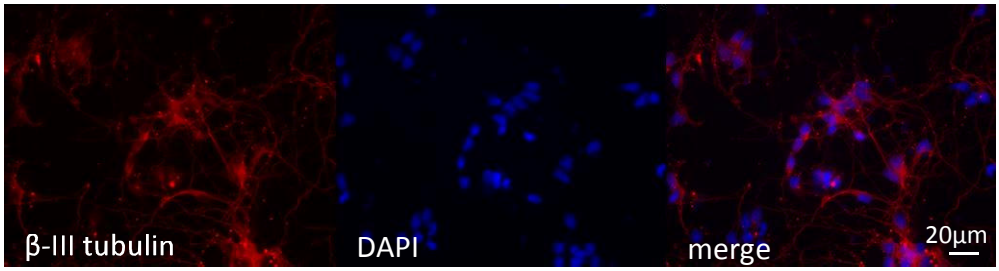

Fig 2-2. Primary rat cortical culture were stained with the neuronal marker, β-III tubulin to clarify the purity of neurons in the culture. More than 90% of DAPI stained nuclei were positive with β-III tubulin in the merge pictures, which indicated a highly purified culture.

2.2 Chemicals

Meclizne was obtained from two different suppliers: Tocris Bioscience (Bristol, UK) and Santa Cruz Biotechnology. In the cell death experiments, meclizine from both suppliers were tested and the protective effect was identical. All other chemicals were obtained from Sigma-Aldrich (St. Louis, US) or Merck Millipore (Darmstadt, Germany) unless otherwise stated.

2.3 Cellular Bioenergetic Analysis

2.3.1 Oxygen Consumption Rate Measurement

2.3.1.1 Principle of Oxygen Consumption Rate Measurement

Oxygen consumption is the essential characteristic of mitochondrial respiration which oxygen converts to water in complex IV. Therefore, oxygen consumption rate (OCR) is recognized as a parameter of mitochondrial respiratory function. XFe extracellular flux analyser, a powerful tool to measure OCR, can detect the tiny change of dissolved oxygen in an isolated, extremely small amount of medium. This technique has been widely used for assessing mitochondrial function (Cantu et al., 2011; Trudeau et al., 2011). Basal OCR is defined as the rate difference between resting status and oligomycin treatment, which inhibits the complex V and block the OXPHOS.

2.3.1.2 Methods of Oxygen Consumption Rate Measurement

Cells were grown on the specific XF24 V7 cell culture microplate with four wells of the plate left empty for calibration. The sensor cartridge was hydrated by calibrant solution and placed into a non-CO_2 incubator overnight before the assay. On the day of the assay, each well of XF24 V7 cell culture plate was washed by warm assay medium three times and then kept in non- CO_2 incubator for 30 minutes to allow equilibration. Meanwhile, the working solution containing different compounds, including glucose, oligomycin, and rotenone were dispensed into each injection port on sensor cartridge to achieve a final concentration at 25mM glucose, 1µg/ml oligomycin and 2µM rotenone in each well. Then the sensor cartridge underwent calibration in the XFe extracellular flux analyser. After calibration, the XF V7 cell

culture microplate replaced the cartridge plate and the assay started. In all the experiments, the protocol (mix/wait/measure cycle) was kept the same. Once the assay had finish, the cells were trypsinised in order to measure the protein concentration for cell number correction.

2.3.2 Extracellular Acidification Rate Measurement

2.3.2.1 Principle of Extracellular Acidification Rate Measurement

Extracellular acidification due to protons releasing into medium is an indirect way to monitor the synthesis of lactic acid, an end-product of glycolysis. The XFe extracellular flux analyzer can also monitor extracellular acidification rate (ECAR) in a real-time manner (Anderson et al., 2013; Blouin et al., 2010). Basal glycolysis was determined by the increase of ECAR after injection of glucose to a no-glucose medium. Afterwards, injection of oligomycin stops mitochondrial respiration and triggers the maximal capability of glycolysis to compensate the energy deficit. Both parameters are vital in investigating the cellular glycolytic flux.

2.3.2.2 Methods of Extracellular Acidification Rate Measurement

Preparations and protocols were identical for the method of OCR measurement (section 2.3.1.2) except for the working compounds in the injection pores. When measuring the ECAR, the sequence of injections was glucose, oligomycin and 2-deoxyglucose (2DG). The final concentration of these compounds in each well was 25mM glucose, 1µg/ml oligomycin and 100µM 2DG.

2.3.3 ATP Synthesis Measurement

2.3.3.1 Principle of ATP Synthesis Assay

Mitochondrial ATP synthesis takes place in the respiratory chain, which is composed of complex I-IV. By providing different substrates and inhibitors, the activity of each complex can be investigated separately. The substrates and inhibitors include glutamate and malate for complexes I, III, IV; succinate and rotenone for complex II, III and IV; and ascorbate and N,N,N',N'-tetramethyl-p-phenylenediamine for complex IV.

2.3.3.2 Methods of ADP Phosphorylation Activity Measurement

The protocols used have been described previously (Gegg et al., 2009). SH-SY5Y cells grown on 10-cm plate were scraped and washed with PBS. Cells were resuspended with the reaction buffer (25 mM Tris, 150 mM KCl, 2 mM K^+-EDTA, 10 mM K_2HPO_4, pH 7.4). An aliquot of cell suspension was mixed with an equal volume of reaction buffer containing 1 mM ADP and substrates (complexes I, III, IV: glutamate+malate (10 µM); complexes II, III, IV: succinate (10 µM)+rotenone (40 µg/ml); complex IV: ascorbate (2 mM)+N,N,N´,N´-tetramethyl-p-phenylenediamine (50 µM)), permeabilized with digitonin, and incubated at 37°C for 20 minutes. The reaction was stopped with perchloric acid, and samples neutralized with 3 M K_2CO_3 dissolved in 0.5 M tri-ethanolamine solution. Debris was removed by centrifugation and ATP measured with the ATP Bioluminesence Assay kit CLSII (Roche, Mannhein, Germany). The cell suspension was also taken for protein quantification to allow cell number correction.

2.3.3.3 Optimization of the Assay

Permeabilization by digitonin is a vital factor in this assay. Insufficient permeabilization leads to less entry of substrates into the mitochondria whereas over-permeabilization breaks down the mitochondrial membrane and results in rupture of the organelle. In order to obtain the most appropriate dosage of digitonin, the cell suspensions were supplied with ADP, glutamate, malate and different concentrations of digitonin, ranging from 0 to 120µg/ml. Figure 2-3 revealed that at concentrations of digitonin within 0-60 µg/ml, there was a positive correlation between the concentration of digitonin with the amount of ATP synthesis. However, the trend was reversed if the concentration of digitonin was more than 60µg/ml. In conclusion, 60 µg/ml digitonin resulted in the best balance between permeabilization and toxicity. Therefore, in the following experiments, this dosage of digitonin was applied universally.

Fig 2-3

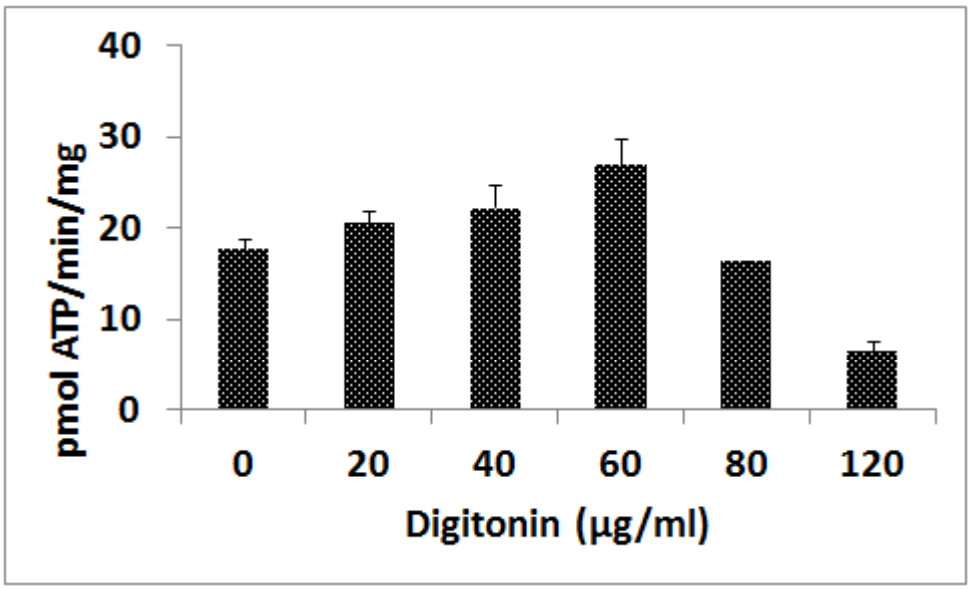

Fig 2-3. The titration of digitonin to obtain the maximum ADP phosphorylation activity from the mitochondrial respiratory chain complex I¬IV. 10µM of glutamate and malate were incubated with cell suspension from SH- SY5Y cells containing different concentration of

digitonin. It revealed that 60µg/ml digitonin gave rise to maximum ATP synthesis whereas 80 and 120µg/ml were likely to induce cell death due to over-permeabilization.

2.4 Protein Quantification by Bicinchoninic Acid Assay

2.4.1 Principle of Bicinchoninic Acid Assay

The bicinchoninic acid (BCA) assay includes two steps. The first step is the reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium. The second step is the colour development reaction. BCA reacts with the cuprous and the chelation of two molecules of BCA with one cuprous results in purple colorization, which can be detected by measuring the light absorbance at 562nm.

2.4.2 Methods of Bicinchoninic Acid Assay

Cell lysates or cell suspensions were mixed with water to a final volume of 50 µl in a 96-well plate. Sample-free wells were used as blank controls while bovine serum albumin (BSA, 0-2000 µg/ml) was used as the protein standard. Kit components A and B (Thermo Scientific, IL, US) were mixed in a 1:50 ratio and 50 µL of the mixture added to each well. Samples were incubated at 37°C for 15~30 minutes depending on the colour change to avoid over-saturation. This colourimetric reaction can be assessed by quantifying the light absorbance at 562 nm using a Synergy HT plate reader (BioTek, Winooski, US). Protein concentrations were determined from the linear absorbance plot generated by measuring known concentrations of BSA protein standards.

2.5 Cell Death Measurements

2.5.1 LDH Release Assay

2.5.1.1 Principle of LDH Release Assay

Cell death was measured by the lactate dehydrogenase (LDH) release assay (Roche, Basel, Switzerland). The principle of this assay is to detect the release of LDH from cytosol to medium. Viable cells keep their cell membrane intact whereas dead cell cannot. The rupture of cell membranes allows the leakage of cytosolic proteins. LDH is a house-keeping protein widely expressed in the cytosol. Once the integrity of cell membrane loss, LDH leaks out and can be detected in the medium. This assay is a relative measurement. Following exposure to TRITON™ X-100, all cells are lysed and the LDH in the medium under these conditions represents the high control (HC). Cell-free medium is used as the low control (LC). All the readings need to subtracted LC and be normalized by HC in order to obtain the percentage of LDH release as a cell death parameter.

2.5.1.2 Methods of LDH release Assay

The protocols used are as described previously (Cleeter et al., 2013). When performing the experiment, cells were plated as groups of wells in the either a 24 or 48-well plate. After 48-72 hours culturing of SH-SY5Y cells or 7 days culturing of rat primary cortical culture, medium were changed to 500µl phenol red-free ones and various treatments were applied for another 24/48/72 hours incubation. On the day of measurement, 50µl of medium was taken out and transferred to the corresponding well in a 96-well plate. Then 50µl of 10% TRITON™ X-100 was added to each well to achieve a final concentration at 1%. After 15 minutes incubation,

50µl of medium contained 1% TRITON™ X-100 were transferred to corresponding wells in the same 96-well plate. The medium were then mixed with the reaction reagents and incubated in room temperature for 30 minutes, before the plate was read at the absorbance 500nm using a Synergy HT plate reader (BioTek, Winooski, US). Results were expressed as a percentage of LDH release using the formula: Percentage of LDH release= (Experiment-LC)*100 %/(HC-LC).

2.5.1.3 Validation of LDH release Assay

It is envisaged that the dosage of toxin is proportional to the degree of cell death and, through the titration of toxin, the proper dosage of toxin which causes substantial but not catastrophic cell loss could be identified. In the present work, two toxins, 6-hydroxydopamine (6-OHDA) and staurosporine were used to induce cell death. In terms of 6-OHDA, an oxidative stress inducer which has been widely used in neurodegenerative models, there was an obvious dose-dependent increase in the percentage of LDH release when the concentration of 6-OHDA was within the range of 30-70µM for 24 hours treatment (Figure 2-4). On this occasion, it also demonstrated the vulnerability of SH-SY5Y cells to 6-OHDA and that 60µM 6-OHDA resulted in substantial but not overwhelming LDH release mimicking the chronic cell loss in neurodegenerative disease (no-toxin: 7.5±1.7, 60µM 6-OHDA: 21.6± 1.7%, n=6).

Another toxin utilized was staurosporine, a protein kinase C inhibitor and an apoptotic inducer. It was used to test the anti-apoptotic theory of the hypothesized neuroprotective effect. Similarly, it revealed a dose-dependent increase in the percentage of LDH released within the range of 10 to 50nM staurosporine for 24

hours treatment. Here, 30nM staurosporine treatment for 24 hours, lead to remarkable but not catastrophic LDH release (no-toxin: 7.9±1.6, 30nM staurosporine: 21.0±0.9%, n=8) (Figure 2-5). Therefore, this concentration of staurosporine was applied to the later experiment to test the anti-apoptotic neuroprotective effect.

These validation experiments also confirmed that the LDH release assay was a suitable test for measurement of the toxin-induced cytotoxicity in the following experiments.

Fig 2-4

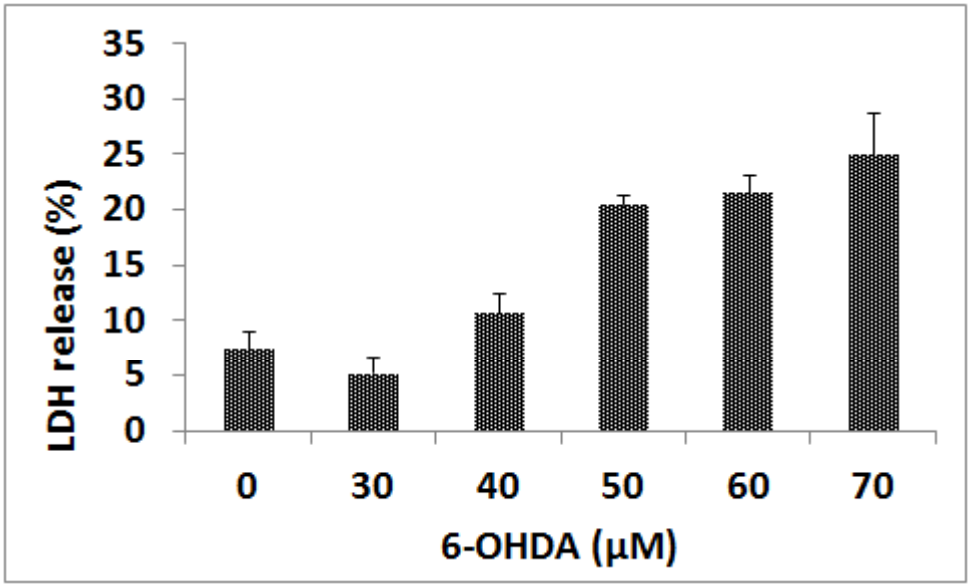

Fig 2-4. The titration of 6-hydroxydopamine (6-OHDA) concentration was used to validate the LDH release assay as a cell death parameters. After 24 hours incubation without 6-OHDA, culturing SH-SY5Y cells exhibited less than 10% of LDH release (7.5±1.7%), which was in line with the microscopic observation. The concentration of 6-OHDA within the range of 30-70μM led to a dose-dependent increase of LDH release (30μM: 5.2±1.6%, 40μM: 10.7±1.8%, 50μM: 20.5±0.9%, 60μM: 21.6±1.7%, 70μM: 25.0±3.8%, n=6). Data were presented as mean±S.E.M.

Fig 2-5

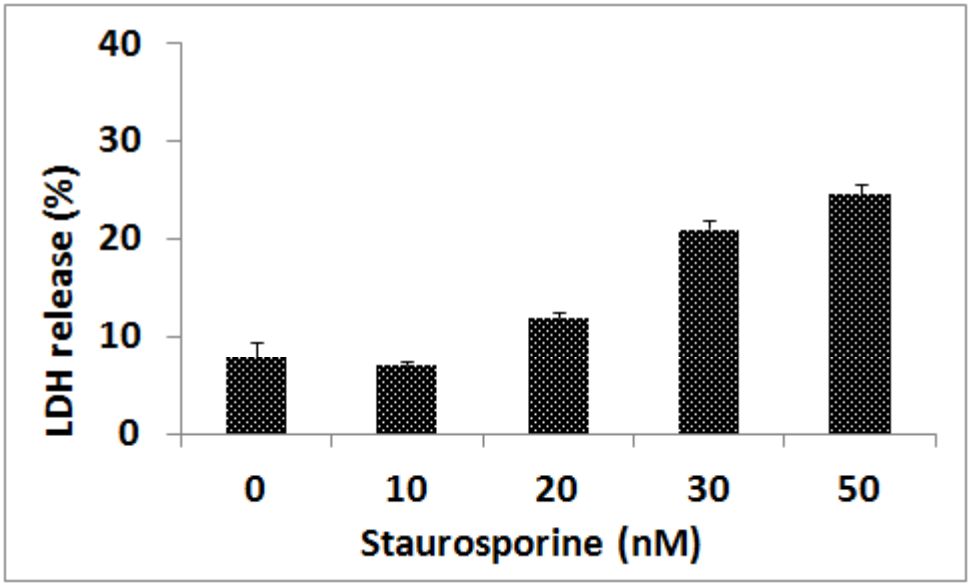

Fig 2-5. The titration of stauropsorine served the same goal as 6-OHDA titration. The concentration of staurosporine within the range of 10-50nM led to a dose-dependent increase of LDH release from SH-SY5Y cells (10nM: 7.1±0.3%, 20nM: 12.0±0.6%, 30nM: 21.0±0.9%, 50nM: 24.0±0.9%, n=8). Data were presented as mean±S.E.M.

2.5.2 Propidium Iodide Binding Assay

2.5.2.1 Principle of Propodium Iodide Binding Assay

Another method of cell death measurement is using the propidium iodide binding assay. Propodium iodide (PI) is an intercalating agent and a fluorescence molecule. When it binds to nucleic acid, the fluorescence excitation maximum is 535 nm and the emission maximum is 617 nm. The principle of this assay, as with the LDH release assay, relies on the cell membrane integrity, as PI cannot penetrate cell membrane, it can only bind to nucleic acid if the cell membrane is disrupted. Again this gives a relative measurement, so, in a group of cells under the same conditions, one or two samples need to be treated with either 1% TRITON™ X-100, in SHSY5Y cells, or 4% paraformaldehyde, in primary rat cortical culture, in order to

obtain a maximum PI fluorescence, and cell-free medium are also required as a background control.

2.5.2.2 Methods of Propodium Iodide Binding Assay

Before performing the experiments, SH-SY5Y cells were cultured in 48-well plate for 2 days and primary rat cortical cultures were prepared in the 24-well plate for more than 7 days. Then the cells were treated according to the different conditions required for another 24 or 48 hours. On the day of measurements, selected wells of cells in each group were treated in order to obtain maximum PI fluorescence. SH-SY5Y cells were treated with 1% TRITON™ X-100 whereas primary rat cortical culture was washed with PBS twice before fixed by 4% paraformaldehyde. Fifteen minutes later, PI was added directly into the medium of all the SH-SY5Y cultures. For primary rat cortical culture, cells were washed with PBS twice, including the pre-fix wells, then stained with 2.5µM PI in PBS. After 15 minutes, the SH-SY5Y cells were measured directly whereas the rat primary cortical culture underwent another PBS washing before measurement. Different processes were required as dead SH-SY5Y cells do not adhere on the bottom of well whereas rat primary cortical culture grow on poly-D-lysine coated cover slips which tend to retain dead neurons. The plates were measured at the fluorescence with 535nm excitation maximum and 617nm emission maximum by a Synergy HT plate reader (BioTek, Winooski, US). Background fluorescence was subtracted from this reading and then the reading was divided by the maximum PI (and multiplied by 100) in order to get the percentage of PI binding as another parameter of cell death.

2.5.2.3 Validation of Propodium Iodide Binding Assay

As a second cell death measuring assay, the PI binding assay should fulfil the same requirement met by LDH release assay: a dose-dependent increase of PI fluorescence. In addition, the cell death reading from PI binding assay should be similar to the former LDH measurement.

Not surprisingly the dosage of 6-OHDA ranging from 20-100μM led to a good relationship between the dosage with the PI fluorescence (Figure 2-6), and 60μM 6-OHDA treated for 24 hours resulted in 28.9±3.2% of PI fluorescence (normalized by the PI fluorescence from Triton X-100 lysed condition), which was very close to the data obtained from LDH release assay. For staurosporine, the pattern was very similar: the concentrations of staurosporine between 10-50nM induced a dose-dependent increase of PI fluorescence (Figure 2-7), and, 30nM staurosporine resulted in 16.0±1.2% PI fluorescence, which was also close to the result measured by LDH release assay.

These validations revealed that both the LDH release assay and the PI binding assay were reliable indicators of cell death, and that the results from each assay were compatible.

Fig. 2-6

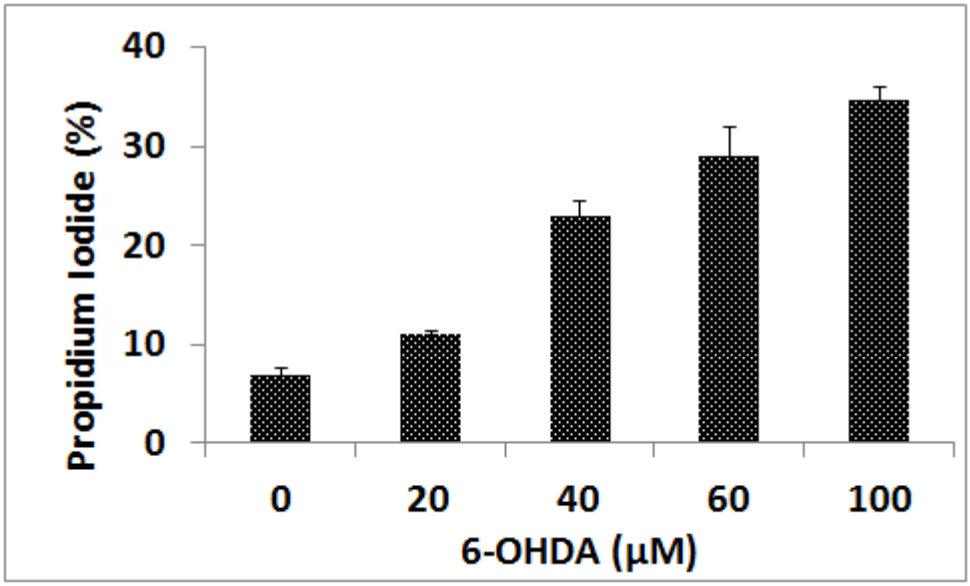

Fig 2-6. The titration of 6-hydroxydopamine (6-OHDA) concentration was used to validate the PI binding assay as another cell death parameters. After 24 hours incubation without 6-OHDA, culturing SH-SY5Y cells exhibited less than 10% of PI fluorescence (6.8±0.8%), which was in line with the LDH release assay. The concentration of 6-OHDA within the range of 20-100µM led to a dose-dependent increase of LDH release (20µM: 10.9±0.7%, 40µM: 22.9±1.7%, 60µM: 28.9±3.2%, 100µM: 34.6±1.4%, n=4). The PI fluorescence was normalized by the PI fluorescence from Triton X-100 lysed condition. Data were presented as mean±S.E.M.

Fig 2-7.

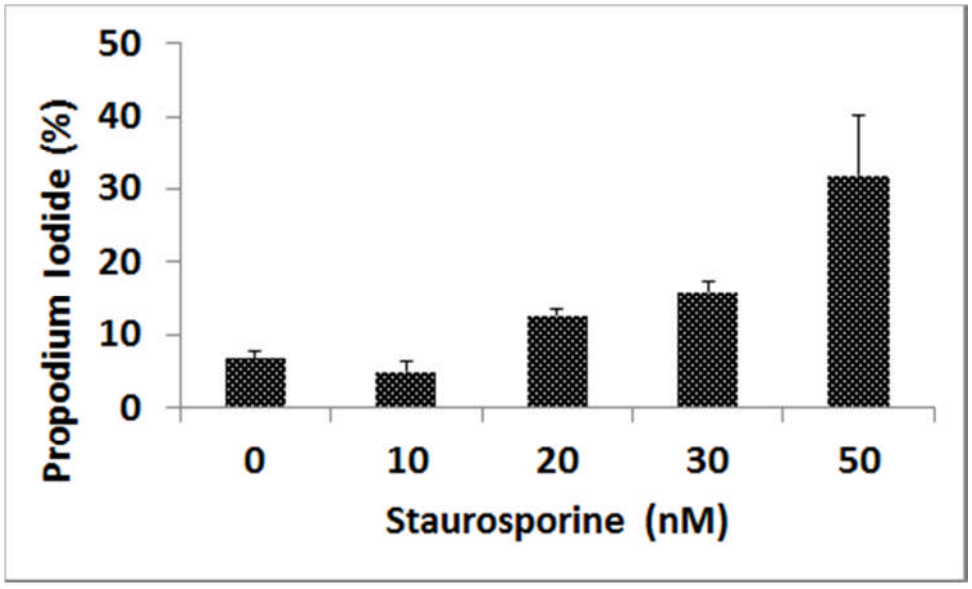

Fig 2-7. The titration of stauropsorine served the same goal as 6-OHDA titration. The concentration of staurosporine within the range of 10-50nM led to a dose-dependent increase of PI fluorescence from SH-SY5Y cells (10nM: 5.0±1.3%, 20nM: 12.8±0.8%, 30nM:

16.0±1.2%, 50nM: 31.8±8.4%, n=4). The PI fluorescence was normalized by the PI fluorescence from Triton X-100 lysed condition. Data were presented as mean±S.E.M.

2.6 Caspase-3 Activity Measurement

2.6.1 Principle of Caspase-3 Activity Measurement

As mentioned in the previous sections, activated caspase-3 is the executor of apoptosis, and the measurement of caspase-3 activity can reflect the intensity of caspase-dependent apoptosis. Caspase-3 has a substrate specificity for the amino acid sequence Asp-Glu-Val-Asp (DEVD). In order to detect the caspase-3 protease activity, the assay contains a specific substrate, rhodamine 110-derived substrate Z-DEVD-R110. The DEVD peptides suppress the fluorescence of R110, however the addition of activated caspase-3 cleaves DEVD leading to the release of a fluorescent R110. Fluorescence of R110 can be detected with peak excitation and emission wavelengths of 496 nm and 520 nm. This fluorescence is continuously measured for 15 minutes, and the rate of increasing fluorescence reflects the activity of caspase-3.

2.6.2 Methods of Caspase-3 Activity Measurement

The protocol for caspase-3 activity measurement has been described previously (Chau et al., 2010). Caspase-3 activity was measured according to the manufacturer's instruction of the EnzChek® Caspase-3 Assay Kit #2 (Life Technologies, Paisley, UK). For the assessment of caspase-3 activity, cells were plated on the 10-cm plate for at least 2 days and treated with either staurosporine, for 4 hours, or 6-OHDA, for 8 hours, before measurement. First the cells were scraped and the resulting cell suspension spun at 4 °C , 21000g for 5 minutes. Then the supernatant was removed and the cell pellets suspended and washed in PBS. After another centrifugation under the same conditions as before, the supernatant

was again removed and the pellets re-suspended in lysis buffer, which contained 10 mM TRIS, pH 7.5, 0.1 M NaCl, 1 mM EDTA, 0.01% TRITON™ X-100 and frozen. Once thawed, supernatant was obtained by centrifugation at 4 °C, 7500g for 5 minutes and 50µl transferred to a well in a black, non-transparency 96-well plate. The reaction mixture was made according to the ratio: 1ml reaction mixture includes 390µl 5 times reaction buffer (50 mM PIPES, pH 7.4, 10 mM EDTA and 0.5% CHAPS), 590µl water, 10µl of 5mM Z-DEVD-R110 and 10µl of dithiothreitol. and 50µl was added to each well. The Z-DEVD-R110 substrate was cleaved by caspase-3 to give rise to fluorescence (excitation/emission, 496/520 nm) which was measured by Synergy HT plate reader (BioTek, Winooski, US) 30 minutes after incubation. The measurement took 15 minutes and the mean velocity of increased fluorescence was recorded. AC-DEVO-CHO caspase-3 inhibitor was applied to certain wells and the readings from those wells represented the non-specific cleavage of substrate.

2.6.3 Validation of Caspase-3 Activity Measurement

In principle, apoptotic inducers are able to increase the caspase-3 activity. However, due to the dynamic nature of apoptosis, a successful monitoring the activation of caspase-3 depends highly on the timing. For example, Gomez-Lazaro *et al* found that the activation of casapse-3 induced by 6-OHDA peaks at 12 hours after treatment and returns to normal at 24 hours(Gomez-Lazaro et al., 2008). Also, some studies had demonstrated many toxins induce apoptosis at lower doses and necrosis at higher doses(Zong and Thompson, 2006). So there are two purposes to the validation. Firstly, the measurement should be able to demonstrate the increase of caspase-3 activity upon apoptotic inducers. Secondly, it should identify

an appropriate dose and duration of toxin treatment to induce the activation of caspase-3.

Figure 2-8 reveals the titration of treatment duration of 500nM staurosporine. Four-hour treatment induced the highest caspase-3 activity compared with 2, 6 and 8 hour treatments. It suggests that 500nM staurosporine treated for 4 hours is superior to longer treatments for the investigation of the intensity of apoptosis. On the other hand, figure 2-9 shows the titration of treatment duration of 100μM 6-OHDA. Caspase-3 activity began to surge after 8 hours after treatment. Therefore, this timing was applied in the following experiments.

Fig 2-8

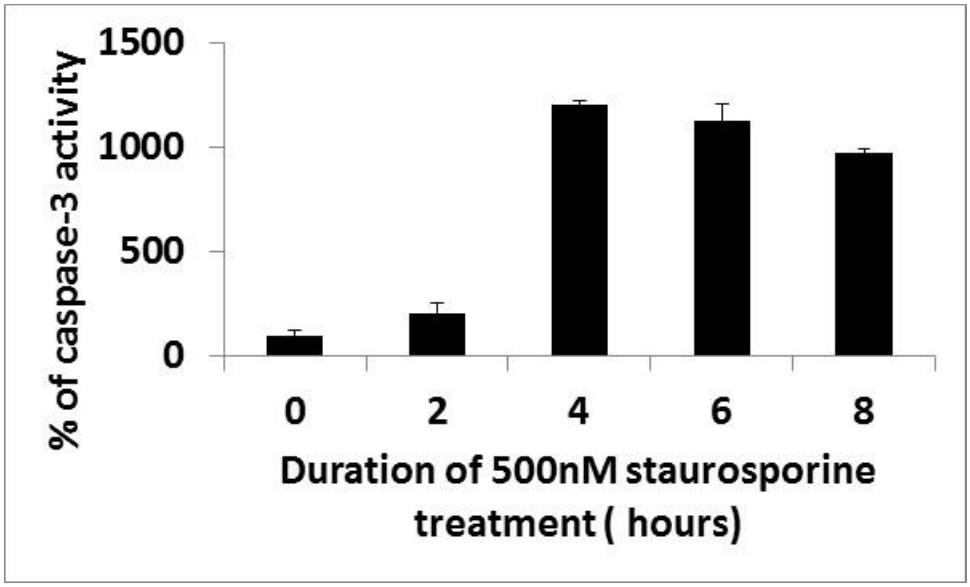

Fig 2-8. The titration of duration of 500nM staurosporine treatment-induced increased caspase-3 activity on SH-SY5Y cells. The caspase-3 activity was normalized by the average activity of non-treated conditions. According the graph, 4 hours treatment activated the most caspase-3 (1206.9±15.5%, n=2) and the activity decreased in 6 and 8 hours treatment. Data were presented as mean±S.E.M.

Fig 2-9

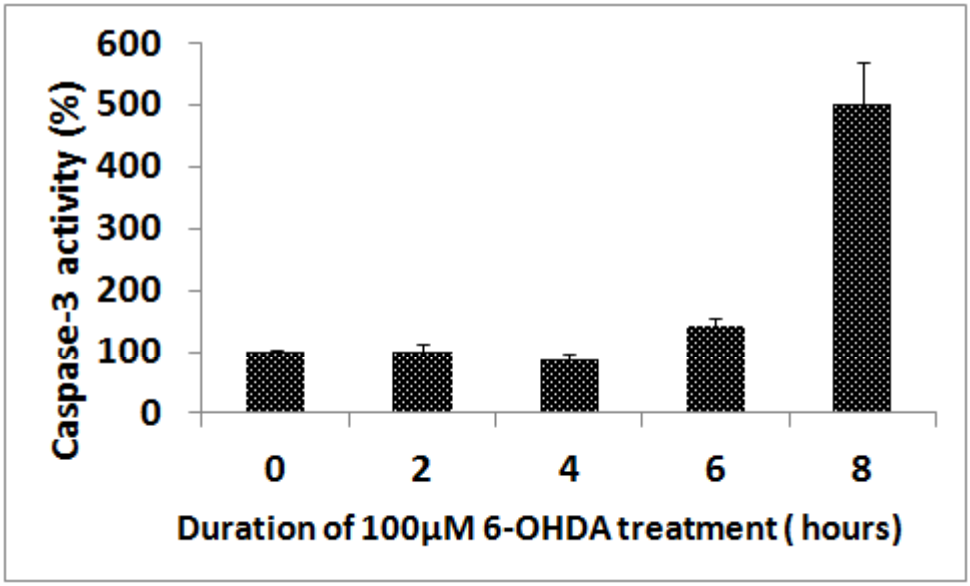

Fig 2-9. The titration of duration of 100µM 6-OHDA treatment-induced increased caspase-3 activity on SH-SY5Y cells. The caspase-3 activity was normalized by the average activity of non-treated condition. According the graph, there was no significant increase of caspase-3 activity between 2-6 hours treatment until 8 hours treatment activated the most caspase-3 (502.1±67.8%, n=5). Data were presented as mean±S.E.M.

2.7 Cytochrome c Release Measurement

2.7.1 Principle of Cytochrome c Release Measurement

In the apoptotic process, MMP leads to the release of mitochondrial apoptotic factors, such as cytochrome c and AIF. In the cytosol, cytochrome c results in the formation of apoptosomes and initiates further caspase activation. By short-term usage of hypotonic cell extraction buffer, the cell membrane will break down and the cytosolic cytochrome c comes out into the bathing buffer, whereas the mitochondrial cytochrome c remains inside the mitochondria, these can then be separated by centrifugation. The amount of cytochrome c present was detected by an enzyme-linked immunosorbent assay (ELISA) kit (Life Techonologies, Paisley, UK). A monoclonal capture antibody specific for cytochrome C is coated onto the wells of the 96-well plate. Both mitochondrial and cytosolic fractions are loaded into

these wells, including a cytochrome c standard. Then, a biotin-conjugated antibody is added which binds to the cytochrome c attached to the first antibody. Streptavidin-HRP is now added which binds to the biotin-conjugated antibody, followed by the substrate solution to form the colored products. The reaction is then terminated by addition of acid and light absorbance measured at 450 nm.

2.7.2 Methods of Cytochrome c Release Measurement

The cells were grown on 6-well plates for a least two days and treated with staurosporine 3 hours before experiment. Following this, the cells were scraped and collected into the medium. The cell suspension was then spun at 21,000g for 5 minutes at 4°C, the supernatant removed and the cell pellet washed by PBS. Then, the pellet re-suspension was spun again under the same conditions as before. After remove supernatant, the cell pellet was re-suspend in cell extraction buffer, (10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 10% glycerol) for 10 minutes, before the suspension received another centrifugation at 11,000g for 5 minutes at 4°C. The supernatant was then transferred to a new eppendorf and represented the cytosolic fraction. The cell pellet was re-suspend by cell extraction buffer plus 1% TRITON™ X-100 for 10 minutes and then spun at 21,000g for 5 minutes at 4°C. This supernatant represented the mitochondrial fraction of cytochrome c. Five microliter of either cytosolic or mitochondrial fraction of supernatant was loaded into the ELISA wells and top-up with another 95 µL of the Standard Diluent Buffer and incubated for 2 hours at room temperature. Different concentration of standard cytochrome c was also loaded to corresponding wells in order to obtain standard curve. After washing wells 4 times, 100 µL of Cytochrome

c Biotin Conjugate solution was added into each well and incubated for 1 hour at room temperature. Then 100 µL of Streptavidin-HRP Working Solution was added to each well and incubated for a further 30 minutes. After washing away Streptavidin-HRP, 100 µL of Stabilized Chromogen was added to each well and incubated for another 30 minutes. The final step was to add 100 µL of Stop Solution to each well which should cause the solution in the wells to change from blue to yellow. The plate was read from the absorbance at 450 nm by Synergy HT plate reader (BioTek, Winooski, US). The absolute cytochrome c concentration was known when the OD from each well was applied to the formula of standard curve. This concentration was also corrected by the protein concentration obtained by the BCA assay.

2.7.3 Validation of Subcellular Fractionation

Appropriate subcellular fractionation is mandatory for detecting the release of cytochrome c from mitochondria to cytosol. Mitochondria and cytosol-specific proteins were used to validate the subcellular fractionation and avoid the possibility of mitochondrial contamination to cytosolic fraction. Fig 2-10 demonstrated that there was no detectable TOM20, a mitochondrial outer membrane protein in the cytosolic fraction, which ruled out the possibility of mitochondrial contamination in the cytosol and indicated the cytochrome c from the cytosolic fraction was purely contributed by the release from mitochondria during apoptosis.

Fig 2-10

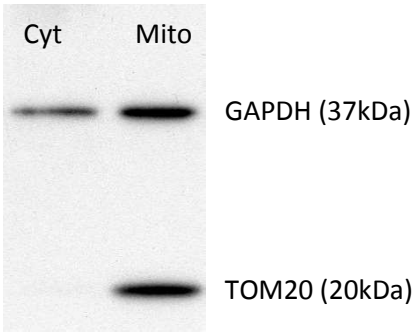

Fig 2-10. Representative western blot for validation of the subcellular fractionation on SH-SY5Y cells. Sample was processed according to the descriptions in the section of 2.7.2. Equal amount of proteins from cytosolic (Cyt) and mitochondrial (Mito) fractions were loaded in to the gel and stained for anti-GAPDH and anti-TOM20 antibodies. There was no mitochondrial TOM20 detected in the cytosolic fraction.

2.8 Mitochondrial Staining by Tetramethylrhodamine, Methyl Ester, Perchlorate (TMRM)

2.8.1 Principle of TMRM Staining

TMRM is a fluorescent derivative of rhodamine 123. It is a cell-permeable and lipophilic cation. Once inside a cell, it accumulates in mitochondria due to the mitochondrial membrane potential and binds to both inner and outer aspects of the inner mitochondrial membrane. The amount of TMRM in mitochondria is proportional to the mitochondrial membrane potential. Measurement of TMRM fluorescence using a fluorescent microscope provides a good indicator of mitochondrial membrane potential and mitochondrial morphology as well.

2.8.2 Methods of TMRM Staining and the Images Acquisition by Confocal Microscopy

Fluorescence of TMRM from cells grown on 22 mm coverslips was measured by real-time confocal imaging as previously described (Duchen et al., 2003; Gandhi et al., 2009). The images were obtained by a Zeiss 510 laser scanning microscope

equipped with an additional Enterprise UV laser source and a cooled charge coupled device camera. The cells were bathed in standard phenol red-free Hank's Buffered Salt Solution at room temperature. Mitochondria were stained by 25 nM TMRM for 30 minutes and the fluorescence was excited using the 543 nm laser line and measured using a 560 nm long pass filter.

2.8.3 Images Analysis: Quantification of Mitochondrial Membrane Potential

Images were analysed by ImageJ software (National Institutes of Health, Maryland, U.S.) following protocols of analysis that have been previously described (Koopman et al., 2005; Koopman et al., 2006). Briefly, raw images were background corrected, linearly contrast optimized, applied with a 7 x 7 'top hat' filter, subjected to twice 3 x 3 median filter, and then threshold, to generate binary images. The background-corrected images were merged with the binary images in order to correct the mitochondrial content and then the average intensity of TMRM staining could be obtained by the histogram analysis.

2.9 Reactive Oxygen Species Measurements by Aconitase Activity Assay

2.9.1 Principle of Aconitase Activity Assay

Aconitase is an iron-sulphur protein and involved in the citric acid cycle, which is responsible for converting citrate to isocitrate. Reactive oxygen species can inhibit aconitase activity by changing the [4Fe-4S] to a [3Fe-4S]cluster within the enzyme. So aconitase activity is a good indicator for cellular oxidative damage. The Aconitase Activity assay is composed of two steps. Firstly, citrate is isomerised to isocitrate by

aconitase, then the isocitrate is converted to α-ketoglutarate in a reaction catalyzed by isocitric dehydrogenase. NADPH is generated in the second step and the assay can be monitored by the rate of NADPH synthesis, measured by the absorbance at 340nm.

2.9.2 Methods of Aconitase Activity Assay

After washing with PBS, SH-SY5Y cells grown on 10cm plate were scraped with and suspended in 500µl distilled water. The 910µl reaction mixture contained cell suspensions with 50 mM Tris-Cl (pH 7.4), 5 mM sodium citrate, 0.6 mM $MnCl_2$, 0.2 mM NADP+ and 1 unit of isocitrate dehydrogenase. This mixture was incubated at 37°C for 20 minutes and then the kinetic increase of absorbance at 340nm was measured. The mean increase velocity was corrected by protein concentration.

2.9.3 Validation of Aconitase Activity Assay

Since aconitase is sensitive to oxidative stress, it is predicted that 6-OHDA, a oxidative stress inducer, would damage the enzymatic activity in a dose-dependent manner. Figure 2-11 reveals that treatment with 6-OHDA at concentrations below 45µM for 24 hours led to a dose-dependent reduction of aconitase activity.

Fig 2-11

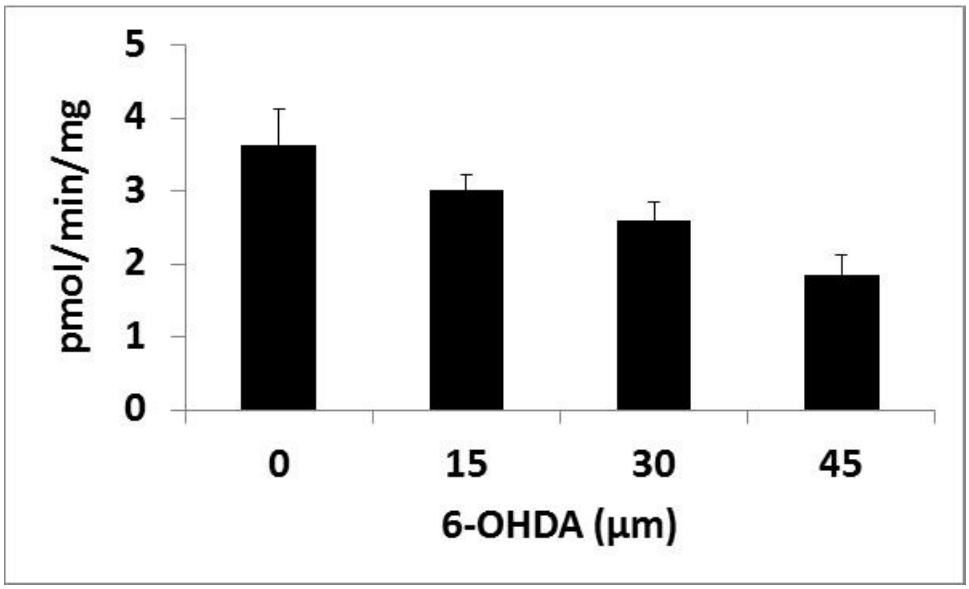

Fig 2-11. Titration of 6-OHDA concentration to validate the aconitase activity assay. SH-SY5Y cells were treated with 6-OHDA for 24 hours at different concentration revealed a negative dose-dependent relationship between the concentration of 6-OHDA with the aconitase enzymatic activity. n=3. Data were presented as mean±S.E.M.

2.10 SDS PAGE

2.10.1 Principle of SDS PAGE

Polyacrylamide gel electrophoresis (PAGE) is a way to separate and analysis proteins. During electrophoresis, the proteins migrate towards the positive electrode through the pores in a polyacrylamide gel. The larger the protein size the more difficult, and therefore slow, the migration. However, if the proteins keep their native status, some higher-order structure may cause the proteins to get trapped in the pores. Sodium dodecyl sulfate (SDS), an anionic detergent, removes all the higher-order structure and turns the sample proteins into their linear form. In addition, SDS also imparts a negative charge to the linearized proteins. With this treatment, the speed the sample proteins run in the gel electrophoresis totally

relies on the electrophoretic mobility, and, in most cases, this mobility is proportional to the size of protein.

2.10.2 Methods of SDS PAGE

Cell pellets were re-suspended in 100µl solution contained 10 mM Tris–HCl pH 7.5, 0.1% of SDS, 1µl of protease inhibitors (Thermo Scientific, IL, US) and 1µl of DNAse1 (Promega, Wisconsin, US) in 10µl enzyme buffer. The cell suspension was incubated at 37°C for 30 minutes. Cell lysate underwent BCA assay for protein quantification. Equal amount of cell lysate was mixed with reducing agent plus sample buffer (both from Life Technologies, Paisley, UK) and incubated at 70°C for a further 10 minutes. The lysates were then separated under reducing condition using Novex 4–12% Bis–Tris gels (NuPAGE, Life Technologies, Paisley, UK) with MOPS SDS Buffer (50 mM MOPS, 50 mM Tris Base, 0.1% SDS, 1 mM EDTA, pH 7.3).

2.11 Western Blot Analysis

2.11.1 Principle of Western Blot Analysis

The purpose of western blot analysis is to detect specific proteins by an immunobloting method. Following gel electrophoresis, proteins with different sizes are separated on the gel. An electric potential can then be used to transfer the proteins onto a polyvinylidene difluoride (PVDF) membrane. The proteins on the PVDF membrane are then blocked by incubation with bovine serum albumin or skimmed milk in preparation for immunoblotting. The immunoblotting includes two steps: firstly a primary antibody binds to the target protein and next a secondary antibody binds to species-specific portion of the primary antibody. The secondary

antibody usually links with horseradish peroxidise, which allows chemiluminescent detection. The luminesce generated by the reaction between the secondary antibody with the substrate can be detected by X-ray film or CCD camera.

2.11.2 Methods of Western Blot Analysis

Following the SDS-PAGE, the resolved proteins on the gel were transferred to PVDF membrane (Immobilon, Millipore, US) using the XCell II blot module (Life Technologies, Paisley, UK). Relative protein mobility was determined using Multi-Mark multi-coloured standard (Life Technologies, Paisley, UK). After blocking for 1 hour with 10% skimmed milk (Sigma–Aldrich, St. Louis, US) in PBS, the blot was incubated with mouse monoclonal anti-HA epitope antibody (clone 16B12, Princeton, US), 1 in 5000 dilution, and rabbit polyclonal anti-β-actin antibody (Ancam, Cmbridge, UK), 1 in 5000 dilution as the loading control, in PBS containing 5% milk powder and 0.2% Tween-20 for 1 hour with shaking. After washing in 0.4% Tween-20, the blot was then incubated with mouse and rabbit IgG conjugated with HRP (Glostrup, Denmark), 1 in 2000–3500 dilution, for 1 hour. This was followed by washes in PBS with 0.4% Tween-20, and chemiluminescence detected using the Amersham ECL Western Blotting Detection Reagent (GE healthcare, Little Chalfont, UK) and Amersham Hyperfilm (GE healthcare, Little Chalfont, UK). The film was developed and signal intensities in the linear range were quantified by the 'Alphadigidoc' software package (AlphaInnotech; San Leandro, USA).

2.12 Immunocytochemistry

2.12.1 Principle of the Immunocytochemistry

Immunocytochemistry is a way to detect the presence and subcellular localization of a target protein in the cell by binding with specific antibody. It requires several steps including: fixation, which immobilises the proteins and the cellular architecture; permeabilization, to allow the antibody access; and blocking, to reduce non-specific antibody binding, followed by primary and secondary antibody binding. The secondary antibody is then conjugated with a fluorescent tag and can be detected by microscope.

2.12.2 Methods of the Immunocytochemistry

Cells cultured on 13mm cover slips were fixed by 4% paraformaldehyde in PBS for 20 minutes in room temperature, followed by permeabilization in methanol at −20 °C for 15 minutes. The cells were then blocked with 1% BSA (Sigma–Aldrich, St. Louis, US) in PBS at 37 °C for 1 hour. For the primary rat cortical culture, the rabbit monoclonal anti-cleaved caspase-3 antibody (Cell Signaling Technology, Massachusetts, US), 1 in 1000 dilution, or mouse monoclonal anti neuron specific β-III tubulin antibody(Abcam, Cambridge, UK), 1 in 250 dilution, with 1% BSA in PBS were added and incubated for 1 hour at 37°C. After PBS wash, the Alexa Fluor 488 goat antirabbit IgG or Alexra Fluor 568 antimouse IgG (Life Technologies, Paisley, UK), both 1 in 200 dilution, were added for another 1 hour at 37 °C. For the SH-SY5Y cells, to confirm the homogeneity of pUL37x1-HA over-expression, the mouse monoclonal anti-HA epitope antibody (clone 16B12, Princeton, US), 1 in 5000 dilution, was used as primary antibody. To determine the intracellular localization

of Bax, mouse Anti-Bax (N-terminus) (Millipore, MA, US), 1 in 500 dilution, was used. Following incubation with the relevant primary and secondary antibodies, the cover slips were washed with PBS and mounting on 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI) dissolved in mounting solution AF1 (CITIFLUOR, London, UK). Images were captured by Axiophot fluorescent microscope (Carl Zeiss, Oberkochen, Germany).

MitoTracker Red CMXRos (Life Technologies, Paisley, UK) is a red-fluorescent dye that stains mitochondria in live cells and is well-retained after aldehyde fixation. MitoTracker Red was added into culturing medium at a final concentration of 6µM and stained for 30 minutes. After this time, the medium was changed with fresh medium for another 30 minutes. Then the cells underwent the fixation and further immunocytochemistry performed as before.

2.13 Fluoro-Jade C Staining

2.13.1 Principle of Fluoro-Jade C Staining

Fluoro-jade C (FJ-C) is a fluorochrome derived from fluorescein, which is highly soluble and acidic. It is a neuron-specific dye that identifies degenerating neurons and can stain several morphological features of neurons including cell bodies, dendrites, axons, and axon terminals(Schmued et al., 1997). It has been widely used for detecting neuronal death in immunohistochemistry assay and some reports also extended the usage to immunocytochemistry(Orellana et al., 2011; Schmuck and Kahl, 2009). However, the real mechanisms of fluoro-jade staining are still unknown

and the involvement of the higher anionic characteristic is suspected by some researchers(Schmued et al., 1997).

2.13.2 Methods of Fluoro-Jade C Staining

The cover slips on which primary rat cortical cultures were grown were fixed with 4% paraformaldehyde for 20 minutes. After 2 washes by PBS, 0.001% of FJ-C solution was added and staining continued for 30 minutes in room temperature. Cover slips were then washed twice with distilled water. Once dry, the cover slips were mounted in mounting solution AF1 (CITIFLUOR, London, UK) with 1 µg/ml DAPI dissolved in it.

Apototic and dead cells on primary cortical culture cells were counted manually. From each cover slip with rat primary cortical culture cells treated with different conditions, 2-4 fileds were selected under 200 times magnification of epi-fluorescent microscope. Each field contained around 75-150 DAPI staining cells. The percentage of apoptotic or dead cells was calculated by the ratio between the number of positive cleaved caspase-3 or FJ-C staining cells to the number of DAPI staining cells. The cell counting was done by single investigator.

2.13.3 Validation of Fluoro-Jade C Staining

An increase in staining should be observed with increasing of toxin provided that the FJC staining is able to detect the dead neurons. Also, the stained-cells should present other nuclear morphological change, such as fragmentation or condensation. Figure 2-12 demonstrates FJ-C staining on the titration of 6-OHDA treatment for 24 hours on rat primary cortical culture cells. There was no obvious

FJ-C stain detected in no-toxin treated cell. 10µM 6-OHDA treatment for 24 hours resulted in some FJC-positive stained cells, and these cells also demonstrated themorphological changes of the nucleus expected in cell death (arrow). 20µM 6-OHDA treatment for 24 hours caused more FJ-C-stained cells. This result indicates the successful cell death detection by FJ-C in rat primary cortical culture.

Fig 2-12

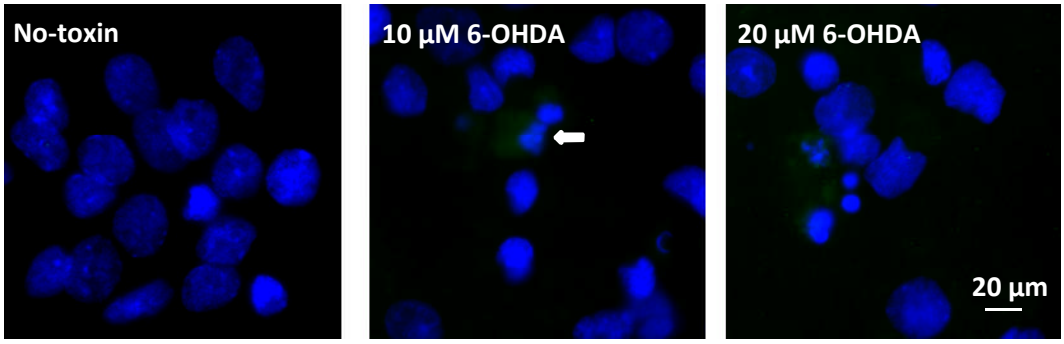

Fig 2-12. The titration of 6-OHDA treatment on rat primary cortical culture to validate the Fluoro-Jade C (FJ-C) staining. No-toxin treatment caused no detectable FJ-C-stained cell. Treatment with 10µM 6-OHDA for 24 hours led to some cells with FJ-C stain. These cells also exhibited nuclear morphological change, which indicated the cells underwent the process of death (arrow). Treatment with 20µM 6-OHDA for 24 hours remarkably increase the number of FJ-C-stained cells.

2.14 Generation of Stable pUL37x1 Over-expression Cell Lines

pUL37x1 cDNA with a 3' haemagluttinin (HA) epitope was cloned into pcDNA3.1 (Life Technologies, Paisley) and transfected (Superfact, Venlo, Netherlands) into normal SH-SY5Y cells. Twenty four hours after transfection, 400µg/ml Geneticin was added to the culture for antibiotics selection. Ninety percent of the cells were lost after the antibiotics selection and the remaining cells formed colonies. When colonies grew to a size more than 5mm they were cloned by cloning cylinders.

2.15 siRNA Silencing

2.15.1 Principle of siRNA Silencing

Small interfering RNA (siRNA) is responsible for RNA interference. siRNA is a double- stranded RNA with a short fragment, 20-25 base pairs, which can be delivered into cell by transfection. In the cytosol, siRNA is cleaved by Dicer, a ribonuclease protein and separated into single strand RNAs, followed by integrated into RNA-induced silencing complex (RISC). RISC can bind with targeted mRNA and halt its translation. Theoretically, any gene can be knocked down by a synthetic siRNA so it is widely used for post-transcriptional gene silencing in a variety of biological experiments.

2.15.2 Methods of siRNA Silencing

In order to get a high efficiency of siRNA transfection, adherent SHSY5Y cells were trypsinsed to produce a cell suspension in the medium before transfection. For each well in a 24-well plate, 1.5µl of 2µM siRNA and 4.5µl of HiPerFect transfection reagent were added to cell suspension. The medium was replaced 24 hours later. The efficiency of silencing was confirmed by western blot analysis.

2.16 Liposomal Transfection

2.16.1 Principle of the Liposomal Transfection

The purpose of liposomal transfection is to deliver genetic material into cells. It serves the same goal as electroporation, calcium phosphate and polymers. However, the advantages of liposomal transfection include less toxicity, high efficiency, ease of use and reproducibility. The principle of transfection is to utilize a

positively charged lipid to wrap a negatively charged nucleic acid and penetrate the negatively charged cell membrane made by the phospholipid bilayer.

2.16.2 Methods of the Liposomal Transfection

The primary rat cortical cultures were grown as described in section 2.1.2.2. The culturing media was changed one day before transfection and each well contained 500µl of medium. For each well of cells, 500 ng of plasmid DNA was added into 50µl Neurobasal medium and 2µl of Lipofectamine™ 2000 (Life Technologies, Paisley, UK) into another 50 µl Neurobasal medium. After 5 minutes of incubation separately, the 2 were combined for a further 30 minutes. Finally, the DNA- Lipofectamine™ 2000 complexes (100 µl) mixture was added directly to each well of the cell containing plates. The incubation took 48 hours and there was no need to remove the transfection reagent.

2.17 Statistics

The sample size (n) in this thesis means the number of "independent replicate experiments"(Lazic, 2010). Before the experiments, cells were cultured in same growing condtion but different and independent wells or dishes. Cell lystes, cell suspension or collected culturing media were kept separated without contamination to each other. All the experiments had been replicated twice or more. One-way ANOVA with post-hoc analysis and two-tailed Student's t-test were used to investigate the difference and *p* value less 0.05 was recognized as significance.

3 Results: Meclizine

3.1 Meclizine Protected SH-SY5Y Cells against 6-OHDA Cytotoxicity

As the hypothesis is neuroprotection, because loss of nigral dopaminergic neurons was the most striking observation of Parkinson's disease brains, it was essential to investigate the protection from cell death conferred by meclizine on the cell line used.

3.1.1 The Protection of Meclizine Was Investigated by LDH Release Assay

It was essential to identify any possible toxicity of meclizine before investigating neuroprotection. A four-time serial dilution from 50µM to 195nM meclizine was tested. The concentration was decided according to previous reports (Gohil et al., 2011; Gohil et al., 2010). The highest concentration (50µM) of meclizine increased the spontaneous death of SH-SY5Y cells whereas 12.5µM to 195nM did not, which was identical with the previous study(Gohil et al., 2010). Control SH-SY5Y cells ,treated with DMSO, induced 6.9±2.3% LDH release after 48 hours incubation, compared with 7.5±1.1% from 195nM meclizine, 5.9±1.0% from 781nM meclizine, 6.9±1.5% from 3.125µM meclizine and 7.0±1.3% from 12.5µM. There was no significant difference between each meclizine treated condition compared with control (Figure 3-1).

6-OHDA is an oxidative stress inducer and has been widely used in neurodegenerative *in vivo* and *in vitro* models. Due to the slowly progressive nature of neurodegenerative disease, treatment of 6-OHDA was given over 48, not 24 hours. 30µM was selected based on the data of validation (Sec 2.5.1.3): 60µM 6-OHDA treatment for 24 hours induced significant cell death on SH-SY5Y cells so half

concentration was tested for twice long treatment. 30μM 6-OHDA treatment for 48 hours induced 20.6±1.2% of LDH release in control SH-SY5Y cells. The concentration of meclizine within the range of 195nM to 12.5μM provided a dose-dependent reduction of LDH release: 17.9±0.7% LDH release from 195nM meclizine simultaneous with 6-OHDA, 16.9±0.8% from 781nM meclizine, 13.3±0.6% from 3.125μM meclizine and 12.2±0.4% from 12.5μM meclizine. The highest two concentrations of meclizine significantly protected cell death compared with control (both $p<0.001$) (Figure 3-1). These results revealed that meclizine at moderate concentration did not increase spontaneous cell death but provided protection on SH-SY5Y cells against 6-OHDA induced cytotoxicity.

Figure 3-1

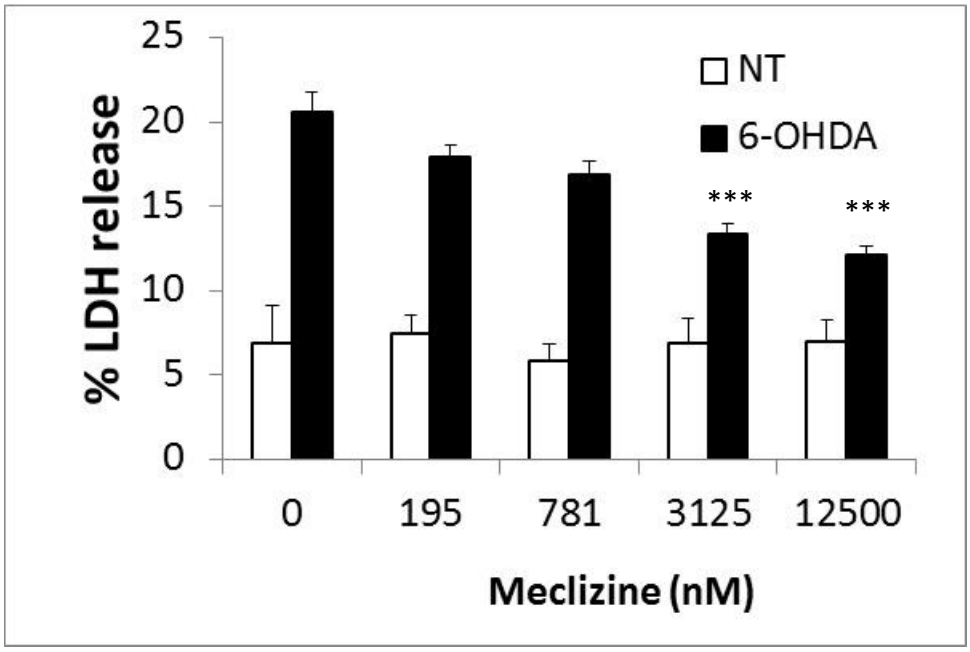

Figure 3-1. Meclizine exhibited a dose-dependent protection against cytotoxicity induced by 30μM 6-OHDA treatment for 48 hours treatment on SH-SY5Y cells. Meclizine was applied at the same time with 6-OHDA. Meclizine at 3.125μM and 12.5μM produced significant protection compared with control (control: 20.6±1.2%, 3.125μM : 13.3±0.6%, 12.5μM:12.2±0.4%, $p<0.001$ respectively, n=8). In addition, dosage of meclizine within this range did not increase spontaneous cell death. Data were presented as mean±S.E.M.

Statistics was performed by one-way ANOVA with Dunnett's post-hoc analysis. (***, $p<0.001$)

3.1.2 The Protection of Meclizine Was Investigated by PI Binding Assay

Another cell death measurement was crucial to confirm the protection of meclizine seen with the LDH release assay. PI, a fluorescent agent, only enters into cells and binds to nucleic acids when the cells lose membrane integrity. PI fluorescence increases 20- to 30-fold when bound to nucleic acids.

The concentration of meclizine within the range of 195nM to 12.5µM provided a significant reduction in PI fluorescence induced by 30µM 6-OHDA treatment for 48 hours, compared with control (control: 25.4±1.1%; 195nM: 20.3±0.4%, $p<0.01$; 782nM: 19.6±1.0%,$p<0.001$; 3.125µM: 20.7±1.2%, $p<0.01$; 12.5µM: 21.6±0.8%,$p<0.05$). In addition, dosage of meclizine within this range did not increase spontaneous cell death (Figure 3-2).

Putting the results of the LDH release and PI binding assays together, moderate concentrations of meclizine clearly demonstrated a consistent protection against 6-OHDA induced cytotoxicity. These findings strongly indicate that meclizine protects against cell death in a neurodegenerative cellular model and proves the hypothesis that meclizine is neuroprotective.

Figure 3-2

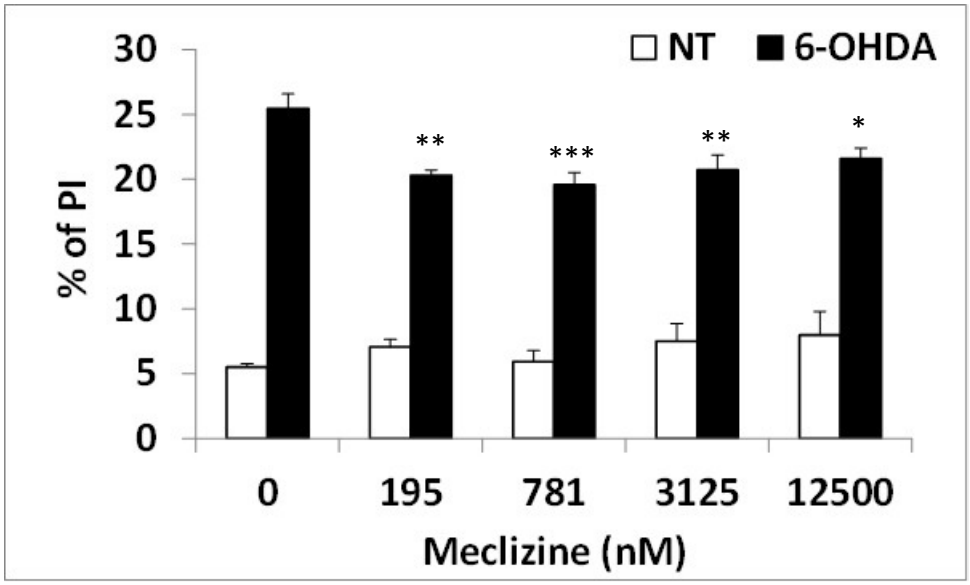

Figure 3-2. PI binding assay demonstrated that the concentration of meclizine within the range of 195nM to 12.5μM significantly protected SH-SY5Y cell death induced by 30μM 6-OHDA treatment foot 48hours treatment compared with control group (control: 25.4±1.1%; 195nM: 20.3±0.4%, $p<0.01$; 782nM: 19.6±1.0%,$p<0.001$; 3.125μM: 20.7±1.2%, $p<0.01$; 12.5μM: 21.6±0.8%,$p<0.05$; n=12). Meclizine was applied at the same time with 6-OHDA. In addition, dosage of meclizine within this range did not increase spontaneous cell death. Data were presented as mean±S.E.M. Statistics was performed by one-way ANOVA with Dunnett's post-hoc analysis. (*, $p<0.05$, **, $p<0.01$, ***, $p<0.001$)

3.2 Meclizine Modulated Mitochondria-dependent Apoptosis

I then sought to identify the mechanism of cell death prevention by meclizine. The hypothesis is that protection of meclizine against neuronal loss is based on modulating mitochondria-dependent apoptosis. This modulation is depended on mitochondrial hyperpolarization, which results from the enhanced glycolysis. This section evaluates the upstream and downstream processes of apoptosis and, as expected, meclizine had no influence on the upstream but showed protection from downstream apoptosis.

3.2.1 Meclizine Did Not Reduce 6-OHDA Induced Oxidative Stress

Mitochondria-dependent apoptosis refers to not only the apoptosis process activated by the release of mitochondrial contents, but also the triggering factors of

apoptosis which depend on mitochondria. ROS are recognized as a by-product of the mitochondrial respiratory chain and excessive ROS are an important factor of apoptosis initiation. In order to exclude the possible suggestion that protection of meclizine results from a ROS scavenging effect, 12.5μM meclizine was applied with and without 30μM 6-OHDA on SH-SY5Y cells. Aconitase activity was measured 24 hours after the treatment. The dosage of 6-OHDA and duration of treatment was based on the data of validation (section 2.9.3): 30μM 6-OHDA treatment for 24 hours induced remarkable decreasing of aconitase activity on SH-SY5Y cells. Compared to control, 12.5μM meclizine had no effect on aconitase activity (control: 2.9±0.2, meclizine: 2.5±0.3 nmole/min/mg, $p>0.05$). 6-OHDA induced the reduction of aconitase activity and 12.5μM meclizine failed to protect the cells from this loss of aconitase activity (control: 2.2±0.1, meclizine: 2.0±0.2 nmole/min/mg, $p>0.05$). Aconitase is vulnerable to oxidative stress and failure to prevent the loss of aconitase activity indicates that the protection of meclizine does not result from ROS scavenging. (Figure 3-3)

Figure 3-3

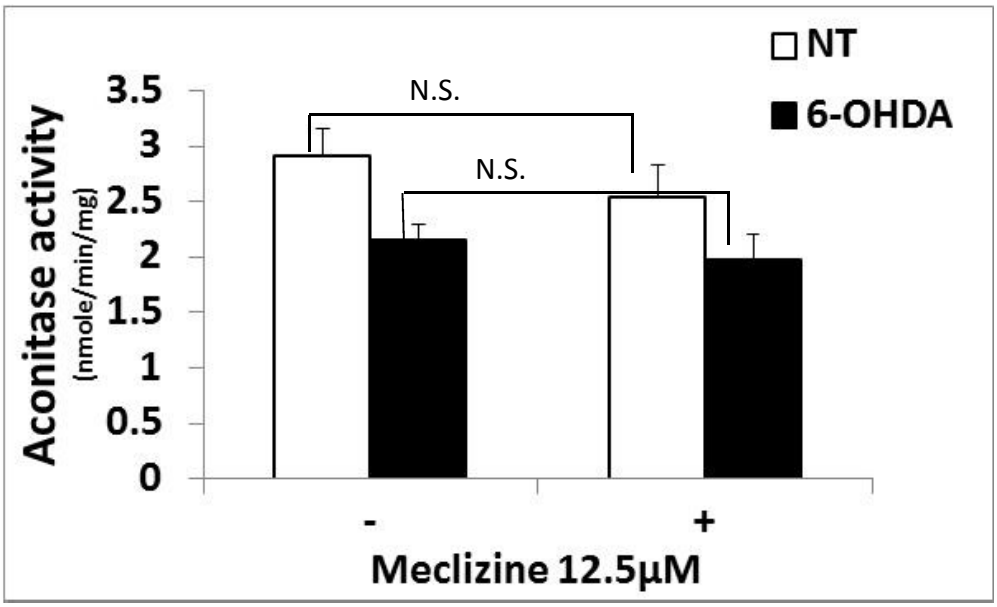

Figure 3-3 12.5µM meclizine, which demonstrated neuroprotection against 6-OHDA induced cell death, did not significantly alter the aconitase enzymatic activity of SH-SY5Y cells in either no toxin or 30µM 6-OHDA treatment for 24 hours groups compared with control (no toxin: 2.9±0.2 from control versus 2.5±0.3 from 12.5µM meclizine, 30µM 6-OHDA: 2.2±0.1 from control versus 2.0±0.2nmole/min/mg from 12.5µM meclizine, n=10). Meclizine was applied at the same time with 6-OHDA. Data were presented as mean±S.E.M. Statistics was performed by two-tailed Student's t-test and *p* value less than 0.05 was recognized as significance. (N.S., non-significant)

3.2.2 Meclizine Hyperpolarized Mitochondria and Rescued the 6-OHDA Induced Depolarization

Mitochondrial hyperpolarization was predicted to be the core anti-apoptotic effect of meclizine. Hence, 12.5 µM meclizine was treated to SH-SY5Y cells 48 hours before the measurement of mitochondrial membrane potential. When no toxin was present, meclizine induced 32% more polarization of mitochondrial membrane potential compared with control (control: 100.0±1.5, meclizine: 132.8±6.1%, $p<0.001$). The mitochondrial membrane potential was adjusted by mitochondrial content and normalized by the average of TMRM fluorescence from control SH-SY5Y cells (Figure 3-4).

Mitochondrial depolarization is believed to be an early process of 6-OHDA induced apoptosis(Lotharius et al., 1999) and meclizine was expected to rescue it to reduce apoptotic cell death. After one hour of 100μM 6-OHDA treatment, the TMRM fluorescence of SH-SY5Y cells dropped more than 20%. Meclizine-treated SH-SY5Y cells maintained normal polarization under 6-OHDA treatment although some degree of TMRM fluorescence reduction was observed (control: 77.0±2.8, meclizine: 101.5±4.0%, p<0.001) (Figure 3-4).

Based on this result, meclizine hyperpolarized mitochondria at basal status, which helped to maintained mitochondrial membrane potential against 6-OHDA induced depolarization.

Figure 3-4

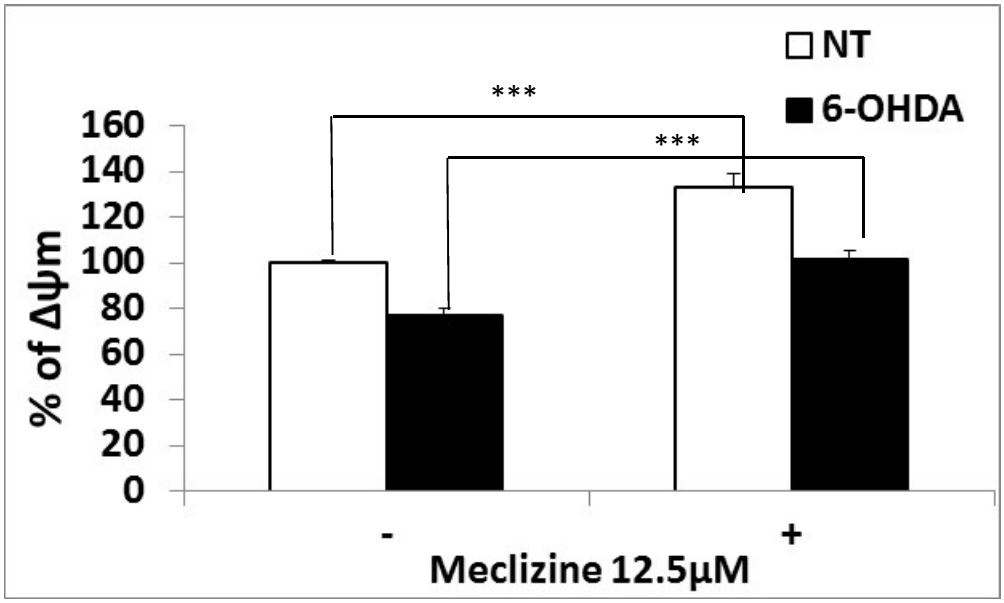

Figure 3-4. Pre-treating SH-SY5Y cells with 12.5μM meclizine for 48 hours significantly hyperpolarized mitochondria (control: 100.0±1.5%, meclizine:132.8±6.1%, p<0.001, n=10) and significantly prevented the mitochondrial depolarization induced by 100μM 6-OHDA treatment for 1 hour (control: 77.0±2.8%, meclizine: 101.5±4.0%, p<0.001, n=10.) Mitochondrial membrane potential (Δψm) was measured by the fluorescence of TMRM with adjustment of mitochondrial content and normalized by the average TMRM fluorescence of non-treated SH-SY5Y cells. Data were presented as mean±S.E.M. Statistics

was performed by two-tailed Student's t-test and *p* value less than 0.05 was recognized as significance. (***, $p<0.001$)

3.2.3 Meclizine Reduced the Caspase-3 Activation Induced by 6-OHDA

Activation of caspase-3 is a late step of apoptosis. The measurement of caspase-3 activity reflects the degree of apoptosis. Several apoptotic inducers, including 6-OHDA, activate caspase-3 whereas anti-apoptotic agents lessen the activation.

With no toxin present, 12.5μM meclizine treatment for 24 hours did not alter the caspase-3 activity compared with control (control: 100.0±5.6, meclizine: 98.7±8.7%, $p>0.05$, caspase-3 activity was normalized by the average of non-treated SH-SY5Y cells). On the other hand, 100μM 6-OHDA treatment for 8 hours, which had demonstrated a significant induced apoptosis and caspase-3 activity from the data of validation (section 2.6.3), increased caspase-3 activity. SH-SY5Y cells pre-treated with 12.5μM meclizine for 24 hours significantly reduced the increase of caspase-3 activity induced by 6-OHDA (control: 1148.0±37.3, meclizine: 937.1±43.4%, $p<0.01$) (Figure 3-5).

It can be concluded that meclizine hyperpolarized mitochondria, and thus prevented 6-OHDA induced depolarization. This effect leads to down-regulation of the downstream apoptosis process and reduces caspase-3 activation. It also demonstrates that ROS scavenging is not responsible for the anti-apoptotic effect of meclizine.

Figure 3-5

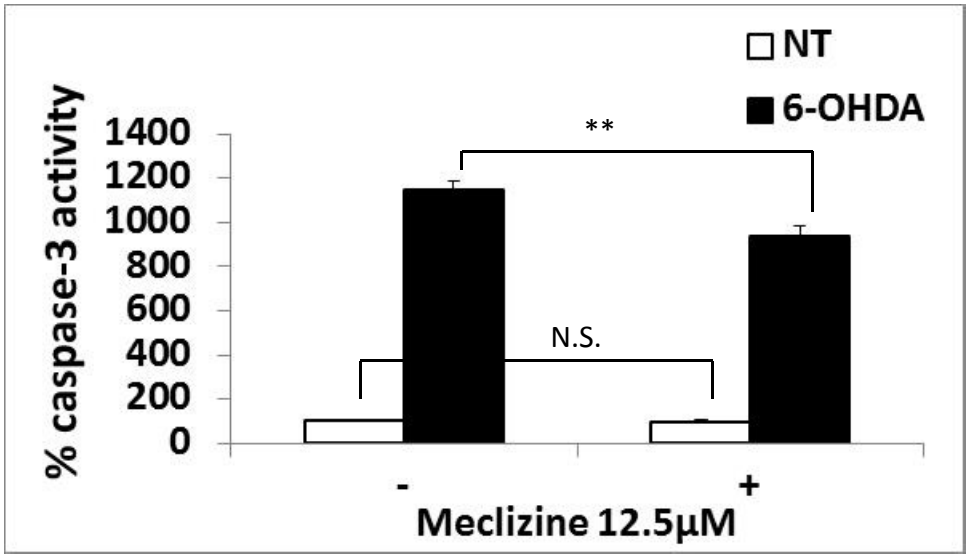

Figure 3-5. Pre-treat with 12.5μM meclizine for 24 hours significantly reduced caspase-3 activity induced by 100μM 6-OHDA treatment for 8 hours treatment on SH-SY5Y cells (control: 1148±37%, meclizine:937±47%, p<0.01, n=7). Caspase-3 activity was normalized by the average of non-treated SH-SY5Y cells. Data were presented as mean±S.E.M. Statistics was performed by two-tailed Student's t-test and p value less than 0.05 was recognized as significance. (N.S., non-significant, **, p<0.01)

3.3 The Protection of Meclizine Was Mitochondrial Hyperpolarization-Dependent

CCCP, a potent mitochondrial uncoupler, was applied to depolarize mitochondria to test the mitochondrial hyperpolarization-dependent protection of meclizine. In both control or 12.5μM meclizine treatment for 48 hours SH-SY5Y cells groups, 1μM CCCP led to significant mitochondrial depolarization (control: 37.4±6.3, meclizine: 39.4±2.2%, p>0.05). The mitochondrial membrane potential was adjusted by mitochondrial content and normalized by the average of TMRM fluorescence from control SH-SY5Y cells (Figure 3-6).

Figure 3-6

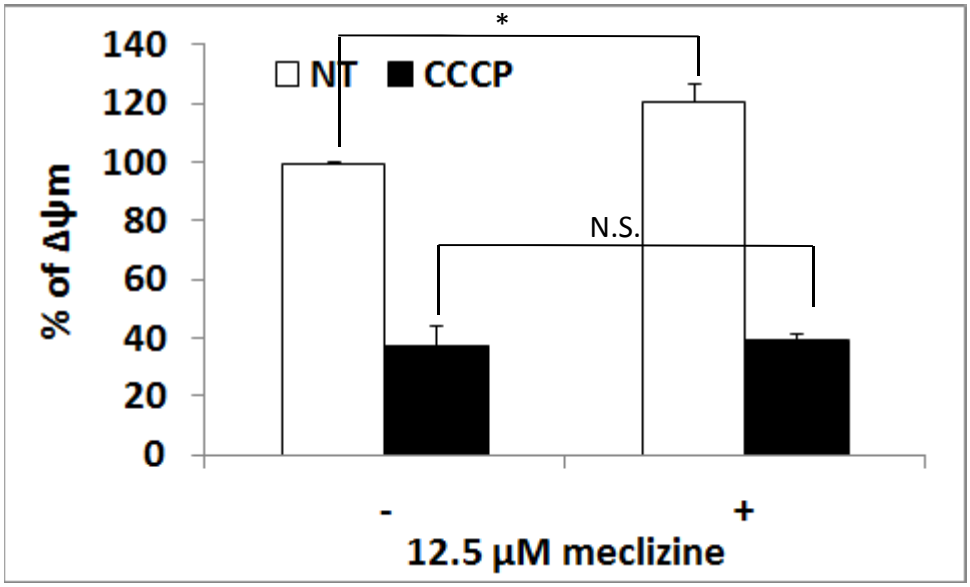

Figure 3-6. Meclizine treatment failed to maintained mitochondrial membrane potential (Δψm) upon CCCP treatment on SH-SY5Y cells. Without CCCP treatment, 12.5µM meclizine treatment for 48 hours significantly hyperpolarized mitochondria (control: 99.3±0.5, meclizine: 120.3±6.4%, n=4, p<0.05). However, for the cells pre-treated with 12.5µM meclizine for 48 hours, 1µM CCCP treatment induced mitochondrial depolarization in both groups without significant difference (control: 37.4±6.3, meclizine: 39.4±2.2%,n=4, p>0.05). Δψm was measured by the fluorescence of TMRM with adjustment of mitochondrial content and normalized by the average TMRM fluorescence of non-treated SH-SY5Y cells. Data were presented as mean ±S.E.M..Statistics was performed by two-tailed Student's t-test and p value less than 0.05 was recognized as significance (*, p<0.05, N.S., non-significant).

It has been reported that CCCP abolishes the mitochondrial membrane potential to cause cell death(Korlipara et al., 2004). Pre-treatment with 12.5µM meclizine for 48 hours failed to prevent the cell death induced by 1µM CCCP treatment for 48 hours compared with control (control: 16.5±1.5, meclizine: 21.6±3.5%, p>0.05) (Figure 3-7).

These results may suggest that the protection of meclizine was mitochondrial hyperpolarization-dependent and there was no further protection from meclizine if the hyperpolarization was ablated.

Figure 3-7

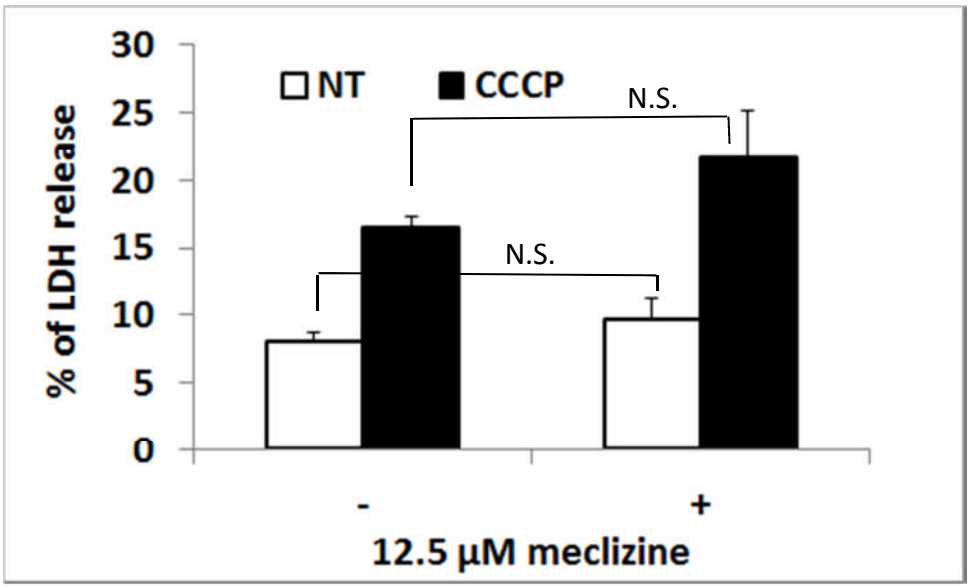

Figure 3-7. Meclizine failed to protect cell death of SH-SY5Y cells induced by CCCP. 1µM CCCP treatment for 48 hours induced 16.5±1.5% of LDH release in control SH-SY5Y cells. Pre-treated with 12.5µM meclizine for 48 hours did not reduce the LDH release (21.6±3.5%, p>0.05, n=8). Data were presented as mean ±S.E.M..Statistics was performed by two-tailed Student's t-test and p value less than 0.05 was recognized as significance (N.S., non-significant).

3.4 The Anti-apoptotic and Cell Death Protective Effect of Meclizine were Glycolysis-dependent.

It was noted that meclizine hyperpolarized mitochondria to modulate apoptosis and protect against 6-OHDA induced cell death in SH-SY5Y cells in section 3.1 and 3.2. Increased glycolysis is thought to be responsible for the protection. However, anti-histamine and anti-cholinergic effects needed to be excluded as meclizine's mechanisms of action and glycolysis inhibitors were applied to confirm the glycolysis-dependent protection.

3.4.1 Meclizine Enhanced Glycolysis in SH-SY5Y Cells , Which Was Attenuated by Glycolytic Inhibitors.

Although Gohil *et al* had demonstrated that 50μM meclizine increased the glycolysis in HEK293, HeLa and STHdh$^{Q7/7}$ cell lines(Gohil et al., 2010), the glycolysis-enhancing effect of meclizine had not been confirmed on SH-SY5Y cells. Glycolysis was measured by XF extracellular flux analyser, which detects small changes of acidification of medium due to lactic acid synthesis, the end-product of glycolysis. SH-SY5Y cells treated with 12.5μM meclizine for 48 hours significantly increased the ECAR compared with control (control: 1405.5±204.3, meclizine: 3613.5±543.6 mpH/min/μg, p<0.01) (Figure 3-8).

In addition, glycolytic inhibitors, 2-deoxy-D-glucose (2DG) and 3-bromopyruvate (3BP) were applied simultaneously with meclizine. Both inhibitors targeted the enzymes responsible for glycolysis. 2DG inhibited hexokinase whereas 3BP inhibited both hexokinase and GAPDH (Cardaci et al., 2012). Co-administration of 10μM 2DG with meclizine for 48 hours demonstrated a trend of down-regulation of glycolysis compared with meclizine alone, and 5μM 3BP significantly lowered the glycolysis induced by meclizine (meclizine: 3613.5±543.6, meclizine+2DG: 2359.0±290.8, p=0.08, meclizine+3BP: 1856.2±332.2 mpH/min/μg, p<0.01) (Figure. 3-8).

These data reveal that 12.5μM meclizine can increase glycolysis in SH-SY5Y cells and glycolytic inhibitors, 2DG and 3BP, attenuate this enhancement.

Figure 3-8

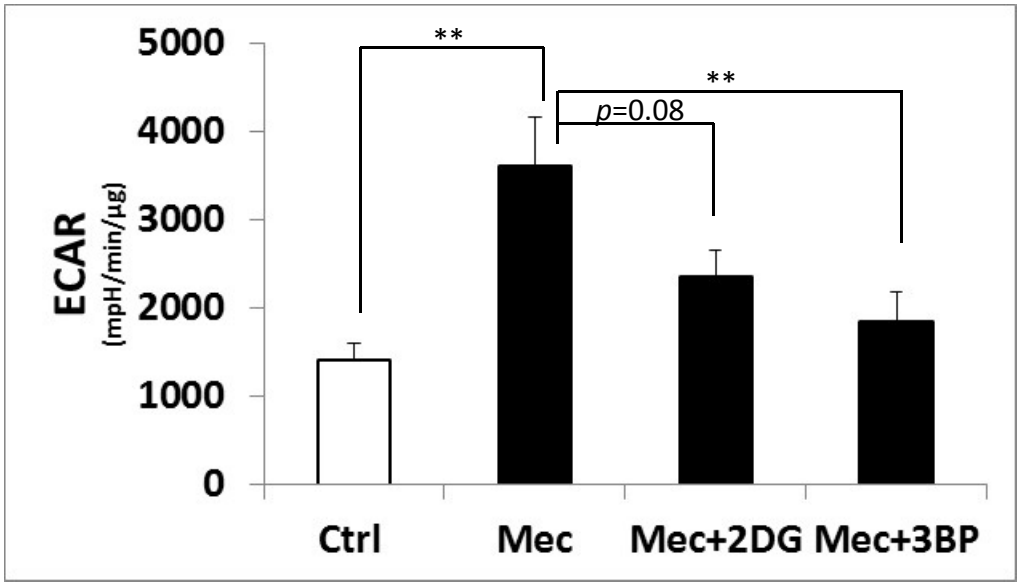

Figure 3-8. 12.5µM meclizine treatment on SH-SY5Y cells for 48 hours significantly up-regulated the glycolytic activity which was measured by ECAR. However, co-administration with glycolytic inhibitor, either 10µM 2-deoxy-glucose (2DG) or 5µM 3-Bromopyruvate (3BP) would markedly reduce the enhancement of glycolysis. (control: 1405±204, meclizine: 3613±544, p<0.01; 2DG: 2359±291, 3BP: 1856±332 mpH/min/µg, p=0.08 and 0.009, respectively, n=6) Data were presented as mean±S.E.M and statistics were performed by one-way ANOVA with Dunnett's post-hoc analysis. (**, p<0.01)

3.4.2 Meclizine Hyperpolarisation of Mitochondria Was Glycolysis-dependent

It has been revealed that meclizine hyperpolarizes mitochondria when no toxin is present. According to previous authors(Cardaci et al., 2012; Iijima, 2006), increased glycolysis leads to mitochondrial hyperpolarization because F_1F_0-ATP synthase consumes glycolysis-generated ATP in order to pump protons out, instead of into, the mitochondria matrix. Glycolytic inhibitors, such as 2DG and 3BP, were expected to reverse the mitochondrial hyperpolarization triggered by meclizine.

Either 10µM 2DG or 5µM 3BP alone for 48 hours did not affect the mitochondrial membrane potential compared with control SH-SY5Y cells (control: 100.0±1.2, 2DG:

96.2±2.8, 3BP: 98.1±1.8%, mitochondrial membrane potential was normalized by the average TMRM fluorescence from control SH-SY5Y cells) (Figure 3-9). However, if cells were simultaneously treated with either 10μM 2DG or 5μM 3BP and 12.5μM meclizine for 48 hours, the hyperpolarization of mitochondria induced by meclizine was significantly reduced (meclizine alone: 135.3±6.2, meclizine+2DG: 106.9±6.9,*p*<0.001, 3BP: 118.8±2.9%, *p*<0.05, mitochondrial membrane potential was adjusted by mitochondrial content and normalized by the average of TMRM fluorescence from control SH-SY5Y cells) (Figure 3-9).

These data support the hypothesis that meclizine induces mitochondrial hyperpolarizaiton by a glycolysis-dependent mechanism.

Figure 3-9

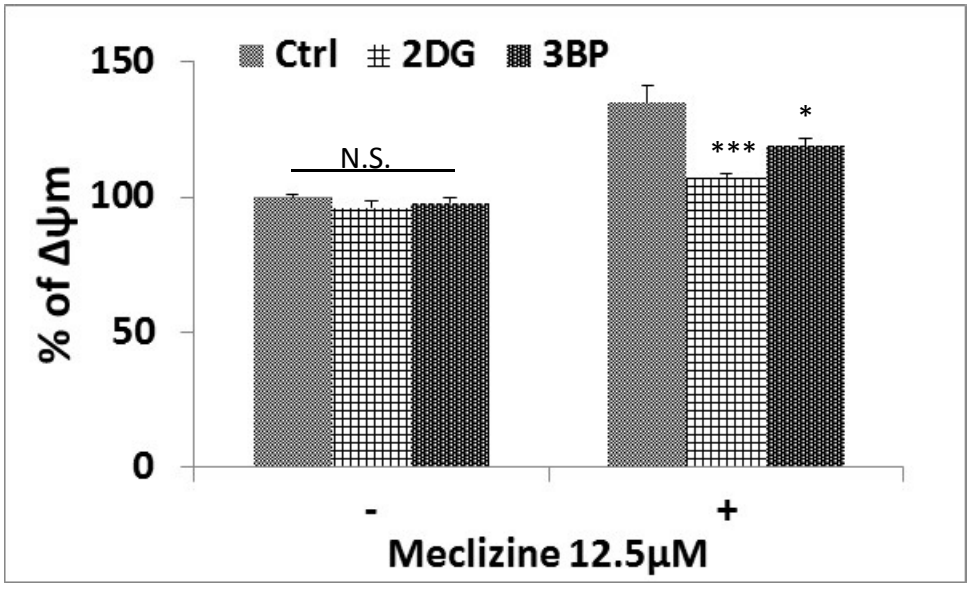

Figure 3-9. 10μM 2DG and 5μM 3BP alone did not affect the mitochondrial membrane potential (Δψm) on SH-SY5Y cells. However, applying either glycolytic inhibitors significantly attenuated the mitochondrial hyperpolarization induced by 12.5 μM meclizine treatment for 48 hours (meclizine: 135±6.2%, meclizine+2DG: 107±1.9%, meclizine+3BP: 119±2.9%, p<0.001 and <0.05, respectively, n=8). Δψm was measured by the fluorescence of TMRM with adjustment of mitochondrial content and normalized by the average TMRM fluorescence of non-treated SH-SY5Y cells. Data were presented as mean±S.E.M and statistics were performed by one-way ANOVA with Dunnett's post-hoc analysis. (N.S., non-significant,*, *p*<0.05, ***,*p*<0.001)

3.4.3 Meclizine Protection of SH-SY5Y Cells against 6-OHDA Cytotoxicity Was Glycolysis-dependent

Section 3.4.2 showed that the anti-apoptotic action of meclizine resulted from glycolysis, but it is important to confirm the association between this glycolysis and cell death protection. Similar to the previous experimental settings, glycolytic inhibitors, 2DG or 3BP, which had been proved to reduce the glycolysis caused by meclizine treatment, were applied with meclizine to investigate the changes in cell death protection capability against 6-OHDA.

In conjunction with 30μM 6-OHDA treatment for 48 hours, neither 10μM 2DG nor 5μM 3BP affected the degree of cell death compared with control (control: 20.1±0.9, 2DG: 21.5±0.4, 3BP: 21.8±2.1%). However, when co-administration with 12.5μM meclizine, both glycolytic inhibitors resulted in significant attenuation of protection against 6-OHDA (meclizine: 11.8±0.3, meclizine+2DG: 16.0±1.2,p<0.05, meclizine+3BP: 25.9±1.3%, p<0.001) (Figure 3-10).

In summary, meclizine increased glycolysis in SH-SY5Y cells and glycolytic inhibitors attenuated this enhancement. Moreover, the key anti-apoptotic mechanism of meclizine, mitochondrial hyperpolarization, was glycolysis-dependent, as was the cell death protection of meclizine against 6-OHDA. These findings support the hypothesis that meclizine modulates mitochondria-dependent apoptosis by increasing glycolysis. These observations will form the basis for further study to determine the relevance of these effects to protect against neuronal cell loss in Parkinson's disease.

Figure 3-10

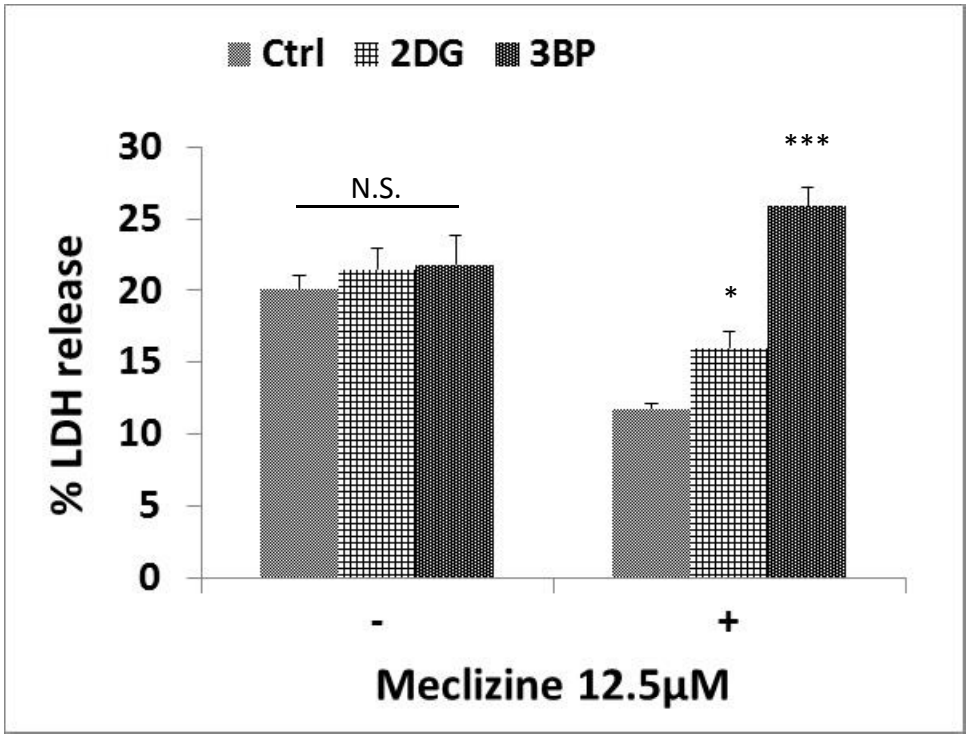

Figure 3-10. The protection of meclizine relied on the effect of increasing glycolysis. Co-administration of either 10µM 2DG or 5µM 3BP, attenuated or reversed the protection of LDH release from meclizine against 30 µM 6-OHDA treatment for 48 hours on SH-SY5Y cells (meclizine: 11.8±0.3%, meclizine+2DG: 16.0±1.1%; meclizine+3BP: 25.8±1.3%, p<0.05 and <0.001, respectively, n=8). Meclizine, 2DG and 3BP were applied the same time with 6-OHDA. Data were presented as mean±S.E.M and statistic was performed by one-way ANOVA with Dunnett's post-hoc analysis. (*, p<0.05, ***,p<0.001)

3.5 Meclizine Did Not Alter the Expression of Glycolytic Enzymes

It is still unknown how meclizine increases glycolysis. HIF-1α is known to increase glycolysis, but it has been proved that meclizine does not increase HIF-1α protein levels(Gohil et al., 2010). In addition, increased expression of glycolytic enzymes can enhance also glycolysis. The present research evaluates the protein expression level of certain key glycolytic enzymes. Figure 3-11 is the representative western blot analysis and Figure 3-12 the result of densitometry analysis. According to the graph and chart, 12.5µM meclizine treatment for 48 hours does not alter the protein level of the target proteins, including hexokinase-1 (HK1), hexokinase-2(HK2),

phosphofructokinase (PFKP) and pyruvate kinase isozymes M1/M2 (PKM1/2). Considering these observations, it remains unclear how meclizine changes cellular glucose metabolism.

Figure 3-11

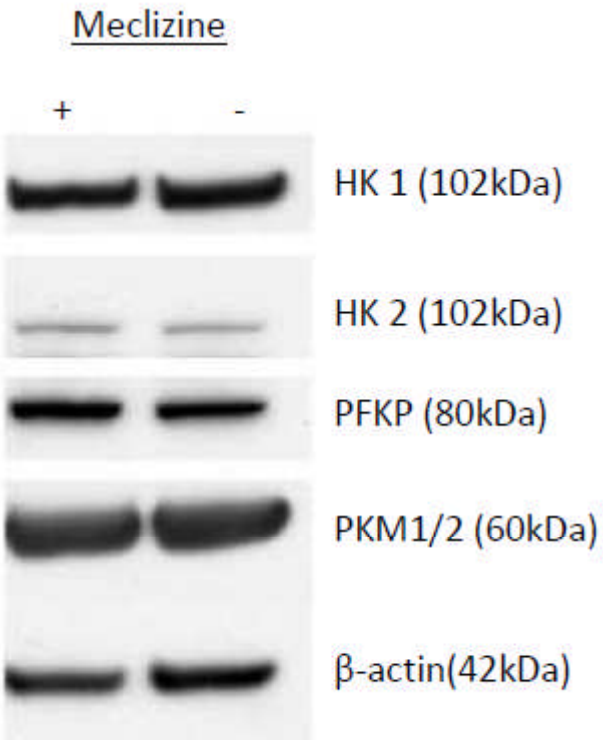

Figure 3-12

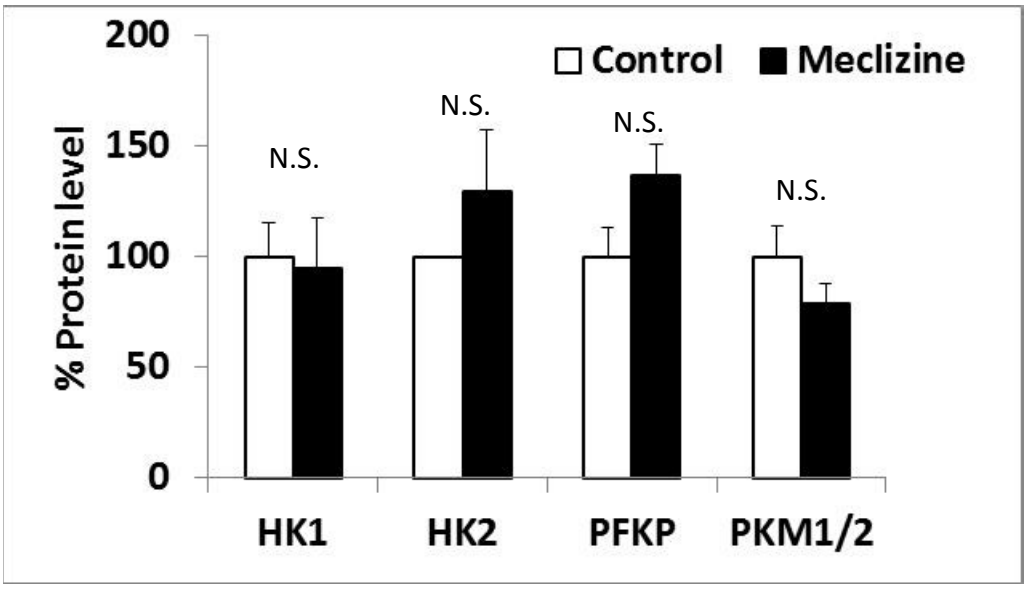

Figure 3-11 and 3-12. Representative western blot and densitometry analysis revealed that meclizine did not alter the protein level of certain key glycolytic enzymes, including hexokinase 1 (HK1), hexokinase 2 (HK2), phosphofructokinase (PFKP), and pyruvate kinase isozymes M1/M2 (PKM1/2) on SH-SY5Y cells. The expression level was corrected by the level of β-actin and normalized by control SH-SY5Y cells. β-actin served as a loading control (n=3). Data were presented as mean±S.E.M. and statistical analysis was performed by two-tailed Student's t-test. (N.S., non-significant)

3.6 Meclizine Protected Rat Cortical Neurons against 6-OHDA Induced Apoptosis and Cell Death

SH-SY5Y is a neuroblastoma cell line which usually exhibits higher glycolysis. However, neurons rely on OXPHOS for ATP synthesis and their glycolytic activity is usually low. Also, neurons are more vulnerable to toxins compared to SH-SY5Y cells so the meclizine-mediated protection seen in SH-SY5Y cells may not be reproducible in neurons. Hence, primary rat cortical cultures were used as another model to test the neuroprotection of meclizine . All the experiments were performed on the matured primary rat cortical cultures, which were cultured for more than 7 days so they presented with obvious neuritics networks.

3.6.1 Meclizine Reduced the Cell Death Induced by 6-OHDA in Rat Cortical Culture

After culturing of the primary rat cortical culture for 7 days, the culturing media were changed to phenol-red free ones for further LDH measuring. The initial concentration of meclizine used was based on the results from SH-SY5Y cells. However, higher concentrations of meclizine (12.5μM) caused increased cell death on primary rat cortical cultures. Therefore, 3.125μM meclizine was applied to test the protection. The concentration of 6-OHDA used was decided according previous literature and the titrating experiments. 10μM 6-OHDA treatment for 24 hours

induced significant but not catasthophic cell loss in primary rat cortical culture so this concentration was chosen.

Compared with control, 3.125µM meclizine treated for 24 hours did not increase the spontaneous LDH release (control: 2.9±0.6, meclizine: 2.3±0.5%). Upon 10µM 6-OHDA treatment for 24 hours, meclizine significantly reduced the LDH release in primary rat cortical culture (control: 10.8±1.4, meclizine: 6.8±0.8%, p<0.05) (Figure 3-13).

These findings show that moderate concentrations of meclizine do not increase spontaneous cell loss, but protected against 6-OHDA induced cytotoxicity in primary rat cortical culture. Therefore, the protection of meclizine on SH-SY5Y cells can be reproduced on primary cells.

Figure 3-13

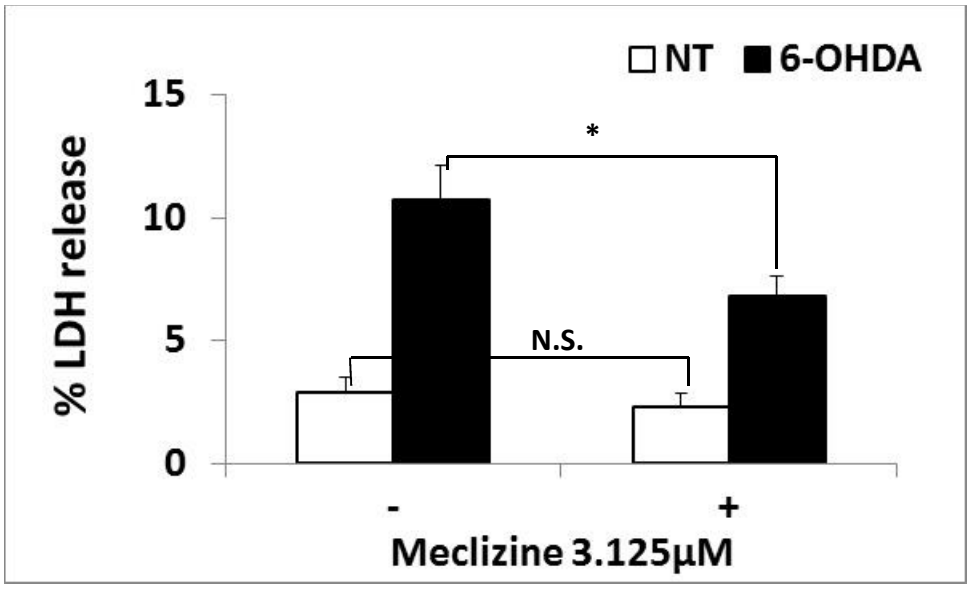

Figure 3-13. On primary rat cortical culture, 3.125µM meclizine presented a significant protection against 10µM 6-OHDA treatment for 24hours induced cytotoxicity measured by LDH release assay (control: 10.8±1.4%,meclizine: 6.8±0.9%,p<0.05, n=8). In addition, meclizine did not increase spontaneous cell death on primary rat cortical culture. Meclizine was applied at the same time with 6-OHDA. Data were presented as mean±S.E.M. and

statistic analysis was performed by two-tailed Student's t-test. (N.S., non-significant, *, $p<0.05$)

3.6.2 The Protection of Meclizine against 6-OHDA Induced Cell Death Was Glycolysis-dependent

It is important to characterise the glycolysis-dependent protection of meclizine on primary rat cortical cultures because previous reports claim that neurons exhibit less glycolysis and fail to up-regulate glycolysis in stress-inducing conditions. In addition, it is necessary to utilise another measurement of cell death to confirm the protection. Here, the PI binding assay is again introduced to meet these two requirements .

When the cultures were free from meclizine, 2DG itself did not increase the cell death upon 10µM 6-OHDA treatment for 24 hours compared with control (control: 11.5±0.7, 2DG: 10.2±1.0%, $p>0.05$). Similar to the results obtained from LDH assay, 3.125µM meclizine significantly reduced the percentage of PI binding while 6-OHDA challenging (control: 11.5±0.7, meclizine: 6.6±0.7%, $p<0.001$). This protection was reversed by co-administration of 10Mm 2DG with meclizine: meclizine with 2DG resulted in a significant higher cell death upon 6-OHDA treatment compared with meclizine alone (meclizine: 6.6±0.7, meclizine+2DG: 19.0±0.9%, $p<0.001$) (Figure 3-14).

These data provide confirmation that first, the protection of meclizine is consistent on primary rat cortical culture and second, although the glycolytic inhibitor itself was not toxic, it was able to attenuate the protection of meclizine. This indicates that the protection of meclizine on primary cells is glycolysis-dependent.

Figure 3-14

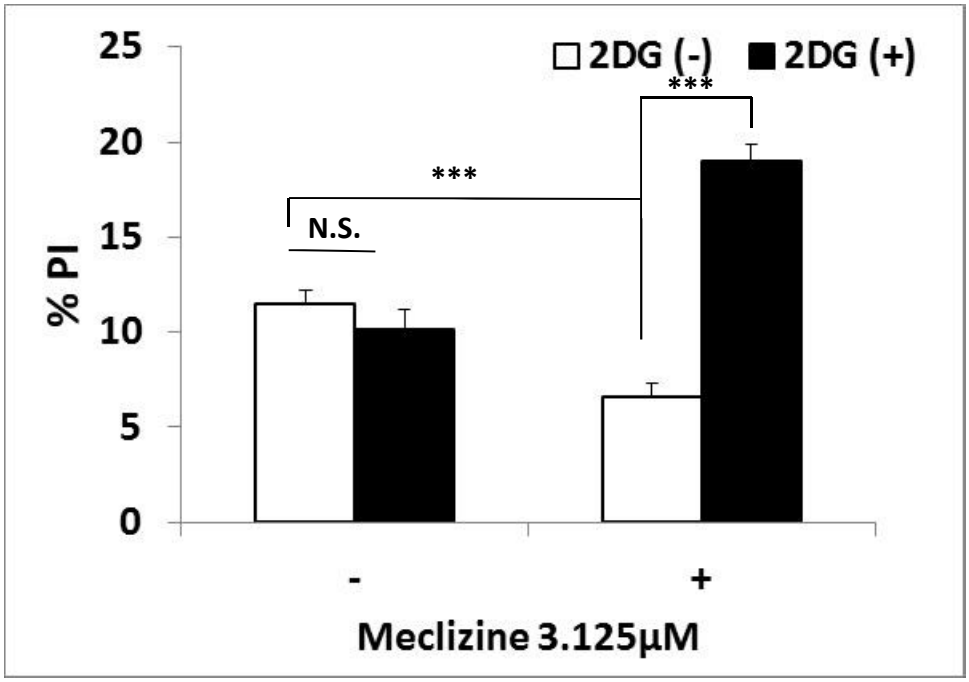

Figure 3-14. The protection of meclizine against 10µM 6-OHDA treatment on rat primary cortical culture cells for 24 hours was confirmed by PI binding assay (control: 11.5±0.7%,meclizine: 6.6±0.7%,p<0.001, n=10). Moreover, although 10µM 2DG itself did not increase the toxicity of 6-OHDA, when meclizine was co-administered with 2DG, the protection of meclizine was lost entirely (meclizie: 6.6±0.7%,meclizine with 2DG: 19.0±0.9%,p<0.001, n=10). Meclizine and 2DG were applied at the same time with 6-OHDA. Data were presented as mean±S.E.M. and statistic analysis was performed by two-tailed Student's t-test. (N.S., non-significant, ***, p<0.001)

3.6.3 Meclizine Reduced Neuron-specific Cell Death Induced by 6-OHDA

Although the primary rat cortical culture has been verified in the previous section, and the percentage of neurons present was more than 90%, it is still arguable that the neuroprotection of meclizine is not genuine because the cultures were not pure. FJ-C, a neuronal degenerative marker was introduced to investigate the absolute neuronal death and protection of meclizine.

In the absence of toxin, 3.125µM meclizine did not increase the percentage of FJ-C positive cells (control: 6.2±0.9%,meclizine: 7.4±0.4%,p>0.05). However, following co-treatment with 10µM 6-OHDA treatment for 24 hours, meclizine significantly

reduced the percentage of FJ-C positive cells in each field(control: 20.4±1.6%,meclizine: 12.7±0.7%,p<0.001) (Figure 3-15).

In summary, meclizine decreases the neuronal death against 6-OHDA in primary rat cortical culture. The mixed culture nature of cortical culture does not affect or distort this protection.

Figure 3-15

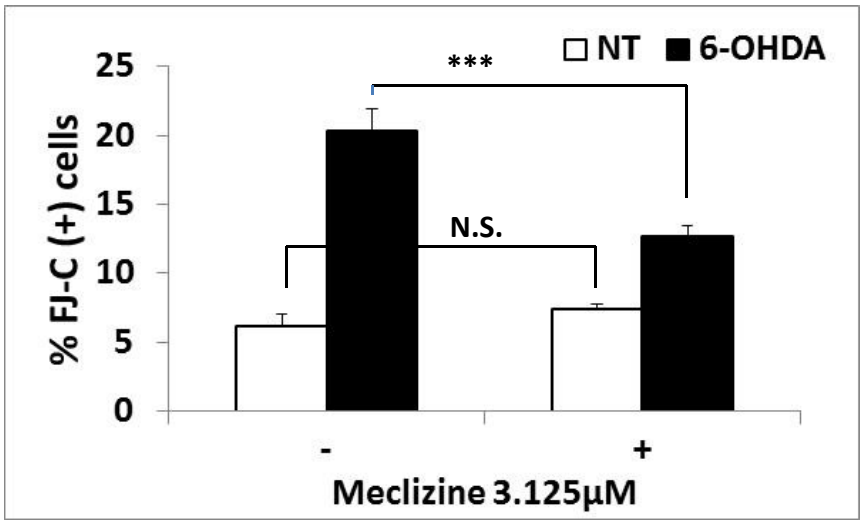

Figure 3-15. On primary cortical culture cells, 3.125µM meclizine treatment for 24 hours did not increase the spontaneous neuronal death but significantly reduced the percentage of FJ-C positive cells against 10µM 6-OHDA treatment for 24 hours (control: 20.4±1.6%, meclizine: 12.7±0.7%, p<0.001, n=10). Meclizine was applied at the same time with 6-OHDA. Data were presented as mean±S.E.M. and statistic analysis was performed by two-tailed Student's t-test. (N.S., non-significant, ***, p<0.001)

3.6.4 Meclizine Hyperpolarized Mitochondria in Primary Rat Cortical Culture

Another important issue, after the demonstration of protection from meclizine in primary rat cortical cultures, was to confirm that meclizine hyperpolarizes mitochondria in these neurons . Figure 3-16 demonstrated that 3.125µM meclizine treatment for 24 hours significantly hyperpolarized mitochondria in primary rat cortical culture (control: 100.0±3.4%,meclizine: 118.5±2.8%,p<0.001, mitochondrial

membrane potential was normalized by the average TMRM fluorescence from control primary rat cortical culture cells). These results indicate that meclizine is able to modulate mitochondrial membrane potential in primary rat cortical culture cells in the same way as SH-SY5Y cells. This hyperpolarization is believed to be responsible for the neuroprotective mechanism of meclizine in neurons.

Fig 3-16

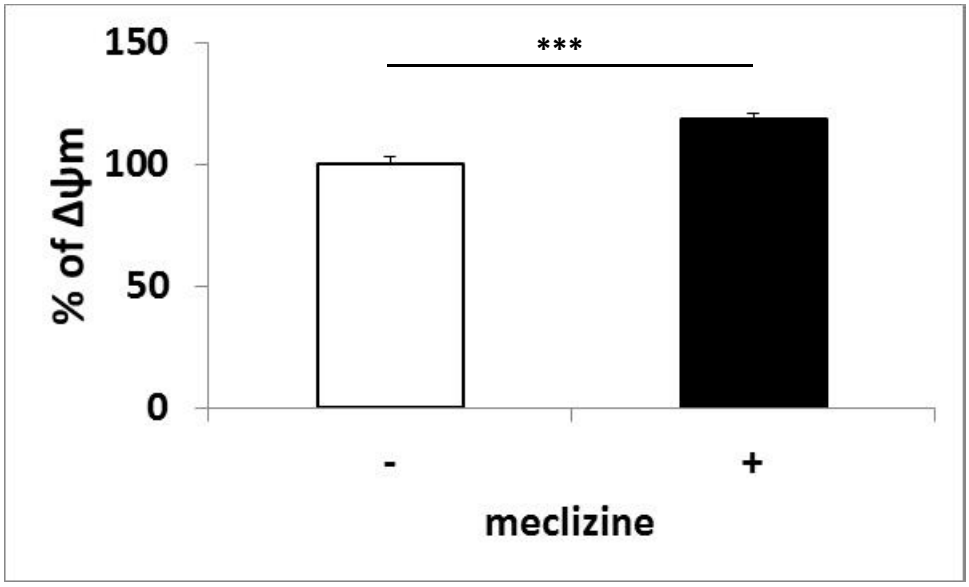

Figure 3-16. 3.125μM meclizine treatment for 24 hours hyperpolarized mitochondria in primary rat cortical culture cells. Measured by TMRM, meclizine significantly produced about 20% more polarization of mitochondrial membrane potential compared with control (control: 100.0±3.4%, meclizine: 118.5±2.8%,p<0.001, n=12, mitochondrial membrane potential was normalized by the average of control primary rat cortical culture cells). Data were presented as mean±S.E.M. and statistic analysis was performed by two-tailed Student's t-test. (***, p<0.001)

3.6.5 Meclizine Reduced 6-OHDA Induced Apoptosis in Primary Rat Cortical Culture

It is predicted that meclizine is able to down-regulate apoptosis and thus reduce the activation of caspase-3, as meclizine hyperpolarized mitochondria in primary rat cortical culture in the same way as SH-SY5Y cells. Primary rat cortical cultures were pre-treated with 3.125μM meclizine for 24 hours and then underwent 20μM

6-OHDA treatment for 6 hours. Coverslips were harvested and went through the process of immuno-cytochemistry. Apoptotic cells were detected by the positive stain of cleaved caspase-3, an intermediate in the apoptotic cascade (Figure 3-17). The fields were randomly selected and the percentage of cells with cleaved caspase-3 stain was recorded. At basal status, 3.125μM meclizine did not alter the percentage of apoptotic cells compared with control (control: 3.9±0.6%, meclizine: 2.0±0.8%,p>0.05). However, when challenged by 20μM 6-OHDA for 6 hours, pre-treatment with 3.125Mm meclizine for 24 hours significantly reduced the percentage of cells with positive cleaved caspase-3 stain (control: 12.4±0.6%, meclizine: 8.8±0.4%,p<0.001) (Figure 3-18).

In conclusion, meclizine presented a similar protection against 6-OHDA in primary rat cortical culture compared with SH-SY5Y cells. It hyperpolarizes mitochondria and is anti-apoptotic as well. In addition, the neuroprotection is glycolysis-dependent. All of these observations support the hypothesis that by enhancing glycolysis, meclizine is able to modulate mitochondria-dependent apoptosis and protect against cell loss in Parkinson's disease cellular models.

Figure 3-17

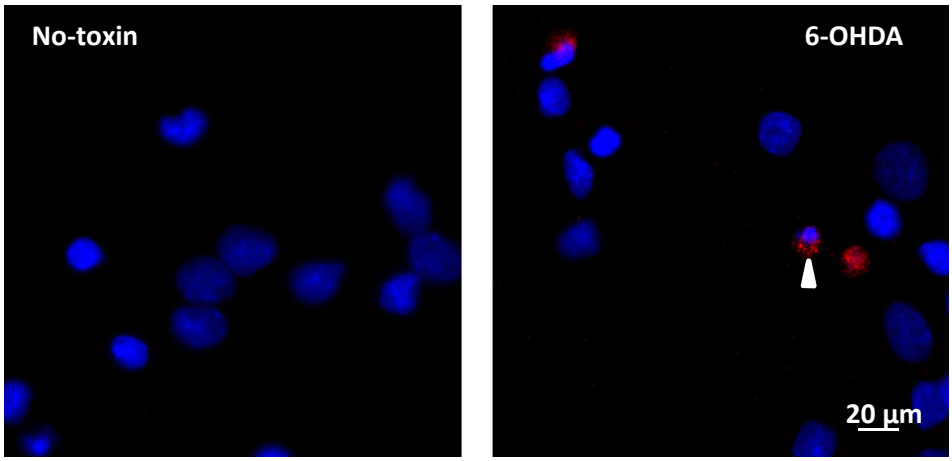

Figure 3-17. Representative images of cleaved caspase-3 staining (red) to detect apoptotic cell death induced by 20µM 6-OHDA treatment for 6 hours on rat primary cortical culture cells. The spontaneous apoptosis was scant (left) and 6-OHDA treatment triggered more apoptotic cell death detected by positive cleaved caspase-3 staining (arrowhead) (right) (blue: DAPI).

Figure 3-18

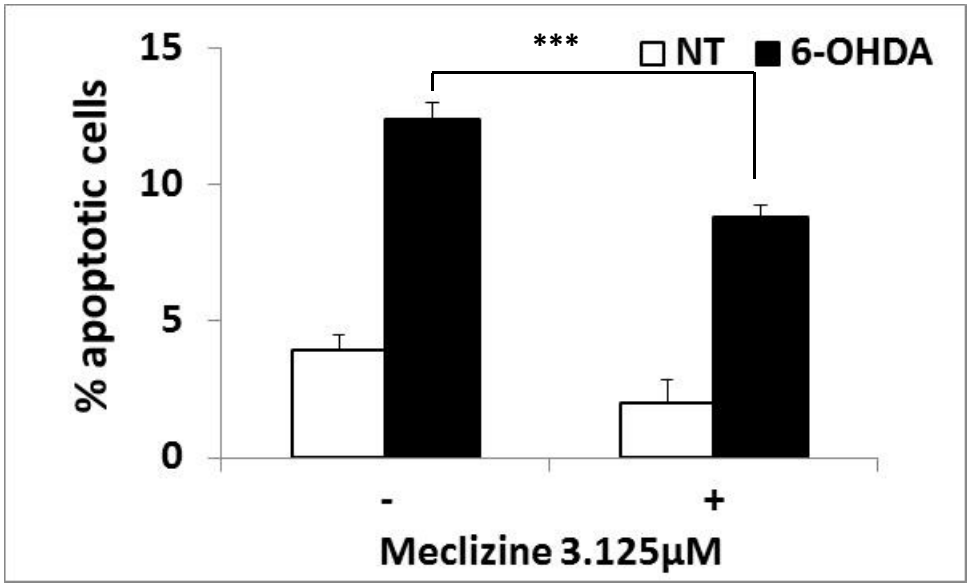

Figure 3-18. Pre-treatment with 3.125µM meclizine for 24 hours significantly decreased the number of apoptotic cells induced by 20µM 6-OHDA treatment for 6 hours on rat primary cortical culture cells (control: 12.4±0.6% versus meclizine: 8.8±0.4%, p<0.001, n=10). Data were presented as mean±S.E.M. and statistic analysis was performed by two-tailed Student's t-test. (***, p<0.001

4 Results-pUL37x1 over-expression

4.1 Generation and Characterization of Stable pUL37x1 Over-expressing SH-SY5Y Cell Lines

4.1.1 Three pUL37x1 Over-expressing SH-SY5Y Cell Lines: Western Blot Analysis Confirmed the Protein Over-expression

Stable pUL37x1 over-expressing cell lines were required to test the hypothesis that pUL37x1 can modulate mitochondria-dependent apoptosis. pUL37x1 cDNA with a 3' haemagluttinin (HA) epitope was cloned into pcDNA3.1 plasmid and transfected into normal SH-SY5Y cells. Transfected cells survived under 400μg/ml geneticin antibiotic selection and formed colonies. Several independent colonies were cloned and maintained under geneticin. Three of these colonies were selected due to the similarities in growing and death characteristics between them and normal SH-SY5Y cells. These colonies are named as pUL37x1-1~3 in the following paragraphs. The whole cell lysate from each cell line was investigated by western blot analysis to detect the levels of pUL37x1-HA protein expression. As well as SH-SY5Y cells, the control group also contained another two cell lines to provide over-expressing controls. The first of these was dsRed-Mito cells. This cell line over-expresses dsRed-Mito, a fluorescent protein which interacts with mitochondria without altering their function. The other control cell line was pcDNA Zero cells, which over-expresses pcDNA3.1(+) plasmid without pUL37x1-HA. In the following sections, dsRed-Mito is labelled as control-2,and pcDNA Zero as control-3. Those two over-expressing cell lines were obtained from Dr. Chau. Data which is labelled as control and pUL37x1 is the combination of each cell line except where specified.

The representative western blot analysis clearly shows that pUL37x1 over-expressing cell lines produced a distinctive band at 60kDa as detected by anti-HA antibody (Figure 4-1). There is no such band detected from either of the three different control lines. Considering the molecular weight of pUL37x1, it is believed that the bands at 60kDa are the expression of pUL37x1-HA from the over-expressing cell lines.

The expression level of pUL37x1-HA in three over-expressing cell lines was analysed by the densitometry method. As no endogenous pUL37x1 protein has been detected in previous reports, the expression level from each line was normalized by pUL37x1-3. Figure 4-2 demonstrated that pUL37x1-1 expressed the most pUL37x1 (192.2±27.7%), followed by pUL37x1-2 (155.4±9.2%) and pUL37x1-3 (100±0%).

Figure 4-1

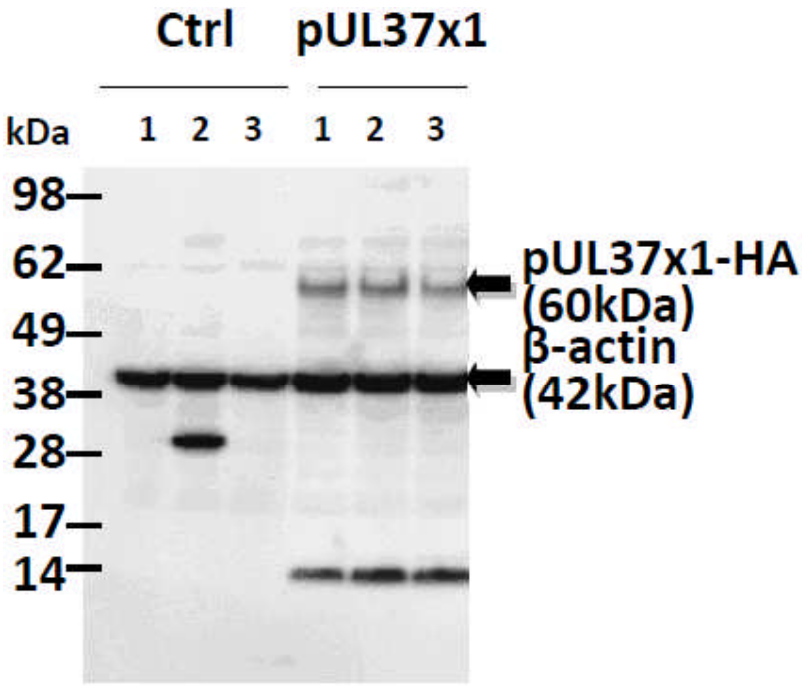

Figure 4-1. Expression construct coding pUL37x1 with an HA-tag at its C-terminus was delivered into SH-SY5Y cells and upon selection with geneticin. Three over-expressing lines were generated. Representative western blot image demonstrated the expression of pUL37x1-HA from three different stable lines of SH-SY5Y. In addition, control-1 was normal SH-SY5Y cell; control-2 was SH-SY5Y cell over-expressing dsRed-Mito, a red fluorescence protein targeted on mitochondrial matrix without altering mitochondrial function; control-3 was SH-SY5Y cells over-expressing pcDNA3.1(+), the empty vector of plasmid. β-Actin was loading control.

Figure 4-2

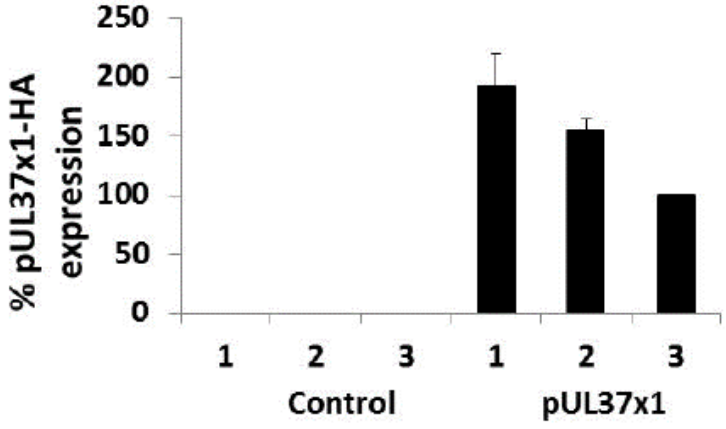

Figure 4-2. Densitometry analysis of pUL37x1 expression level from three pUL37x1 over-expression lines revealed pUL37x1-1 line expressed the highest level of pUL37x1 (192.2±27.7%), followed by pUL37x1-2(155.4±9.2%) and pUL37x1-3 (100±0%)(n=6). The expression level from each line was normalized by pUL37x1-3 and corrected by β-actin. All control cells did not express detectable pUL37x1-HA. Data were presented as mean±S.E.M.

4.1.2 Three pUL37x1 Over-expressing SH-SY5Y Cell Lines: Immunocytochemistry Confirmed the Homogenous Over-expression in Three Cell Lines

Western blot analysis demonstrated the ectopic expression but failed to establish the homogeneity of pUL37x1 over-expression. As well as antibiotic selection, to confirm the purity of ectopic expression required for the immuno-cytochemistry assay. In SH-SY5Y cells, there was no detectable pUL37x1-HA. In contrast, most of the cells in pUL37x1 over-expressing lines exhibited a positive pUL37x1-HA staining (Figure 4-3).

Figure 4-3

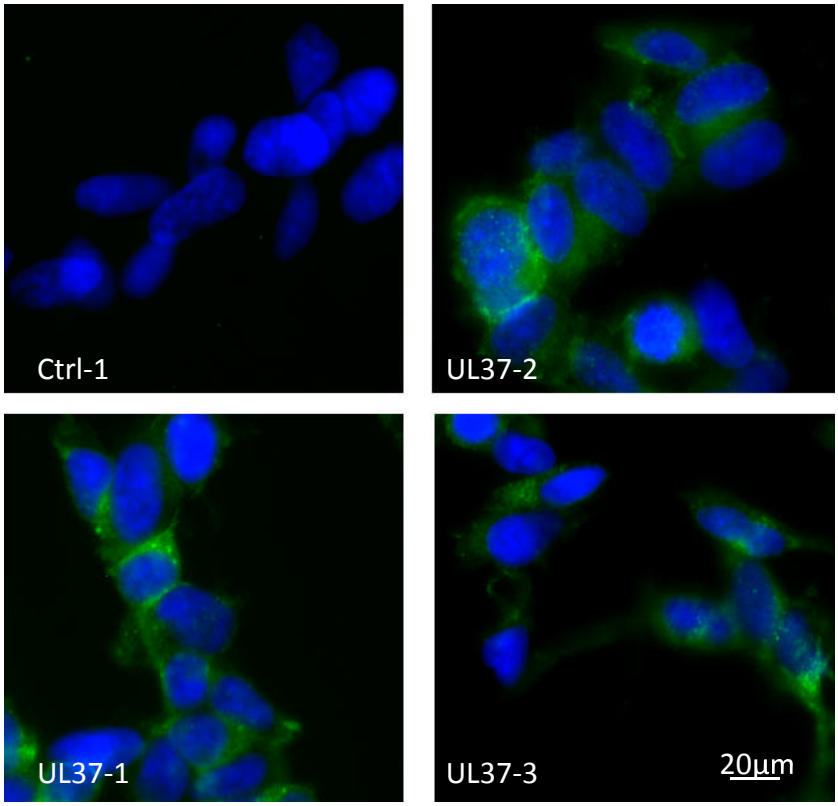

Figure 4-3. All three pUL37x1 over-expressing SH-SY5Y cell lines homogenously expressed pUL37x1-HA which was detected by immunocytochemistry whereas the control SH-SY5Y cells did not express detectable pUL37x1-HA (green: pUL37x1-HA, blue: DAPI).

4.1.3 Mitochondrial Localization of pUL37x1

It has been mentioned in the previous sections that the mitochondrial localization of pUL37x1 is highly associated with the anti-apoptotic effect. A confocal microscope with a small pinhole provided narrow depth-of-field imaging, thereby clearly demonstrating the co-localization of pUL37x1 with mitochondria (Figure 4-4). In the representative image, plenty of pUL37x1-HA (green) is obviously co-localized with MitoTracker (red), a mitochondrial marker.

Figure 4-4

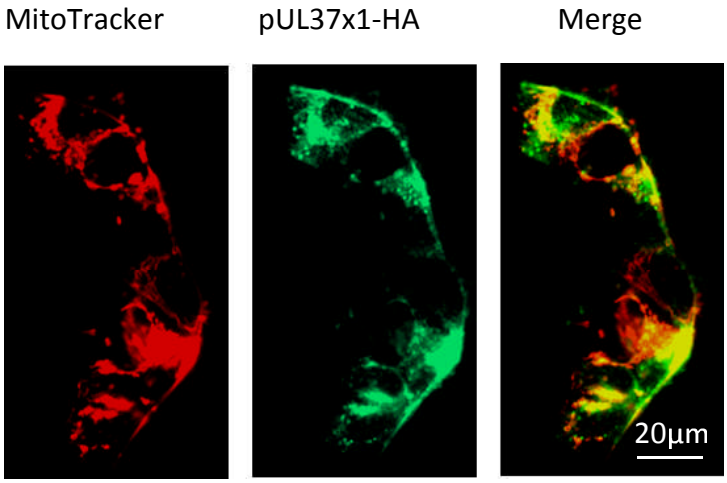

Figure 4-4. The mitochondrial localization of pUL37x1 from pUL37x1 over-expressing SH-SY5Y cells. In the representative image, mitochondria were labelled by MitoTracker (red) and pUL37x1-HA was detected by anti-HA antibody (green). In the merged image, it obviously demonstrated plenty of co-localization (yellow).

To summarise, three independent stable pUL37x1 over-expressing SHSY5Y cell lines were generated. All these cell lines expressed a significant amount of pUL37x1-HA compared with control. In addition, the antibiotic selection process resulted in a high purity of over-expression. The over-expressing proteins also exhibited mitochondrial co-localization.

4.2 pUL37x1 Over-expression Protected Against Toxin-Induced Cytotoxicity

Two cytotoxic agents were applied to SH-SY5Y cells in order to test the neuroprotection of pUL37X1. 6-OHDA, an oxidative stress inducer, is a common toxin used in neurodegenerative *in vivo* and *in vitro* models to induce neuronal death. Another toxin is staurosporine, a protein kinase C inhibitor. Staurosporine is a strong apoptosis inducer and has been widely used to challenge the anti-apoptotic effect of neuroprotective agents. pUL37x1 over-expression was expected to reduce the cell death induced by 6-OHDA and staurosporine in SH-SY5Y cells.

4.2.1 pUL37x1 Over-expression Protected Against 6-OHDA Induced Cytotoxicity

4.2.1.1 The Protection Was Investigated by LDH Release Assay

Stable pUL37x1 over-expressing cell lines do not demonstrate increased spontaneous cell death compared with control (control: 6.5±0.7%, UL37: 5.8±0.4%,p>0.05). Treatment with low doses of 6-OHDA (30μM), in pUL37x1 over-expression cell lines did not demonstrate protection compared with control (control: 9.1±0.8%, pUL37x1: 7.5±1.1%, p>0.05). This failure resulted from the sub-lethal dosage used and treatment time, which only induced less than 10% LDH release in control. However, when challenged with higher doses of 6-OHDA (60μM) for 24 hours, pUL37x1 over-expression significantly reduced the percentage of LDH release (control: 30.6±1.9%, UL37: 12.9±0.6%, p<0.001) (Figure 4-5).

The protection of pUL37x1 over-expression also worked following longer toxin exposure times. Treatment with 30μM 6-OHDA for 48 and 72 hours, in pUL37x1 over-expression cell lines resulted in significantly reduced cell death compared with

control (48 hours, control: 22.8±1.5%, UL37: 15.1±1.9%, p<0.01, n=8; 72 hours, control: 30.2±1.3%, UL37:15.1±0.6%, p<0.001, n=6) (Figure 4-6). In addition, the protective trend was consistent in all three lines. There was no relationship between protection and the pUL37x1 expressing level (supplemet). The dosage and duration of 6-OHDA treatment was mainly based on the data of validation (Sec 2.5.1.3).

These results support the hypothesis that pUL37x1 over-expression is neuroprotective and also provides evidence that over-expression itself is not toxic.

Figure 4-5

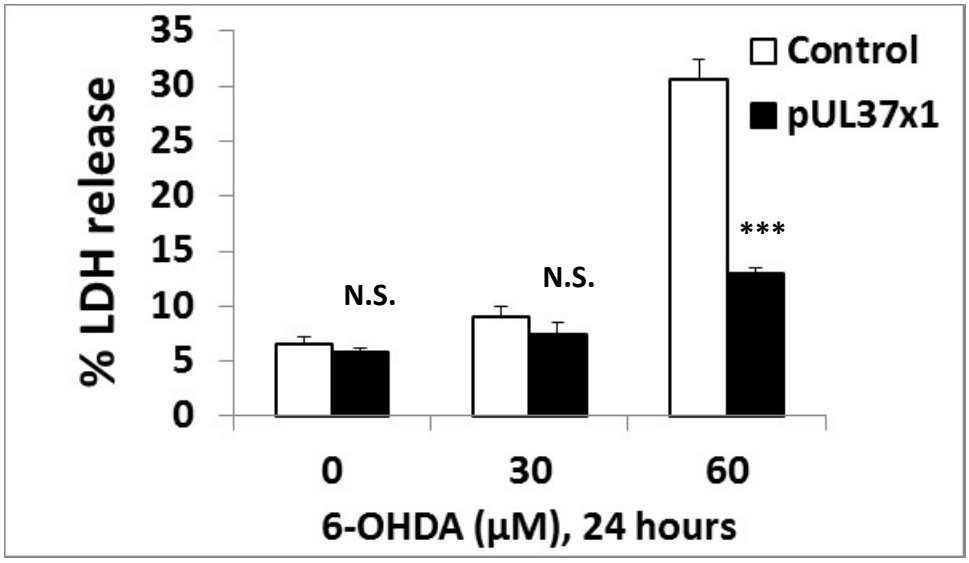

Figure 4-5. pUL37x1 over-expression did not increase spontaneous cell death on SH-SY5Y cells compared with control and significantly decreased the percentage of LDH release induced by 60µM 6-OHDA treatment for 24 hours (30.6±1.9% in control, 12.9±0.6% in pUL37x1 over-expression, p<0.001, n=6). Data were presented as mean±S.E.M. Statistics was performed by two-tailed Student's t- test. The n numbers represented the experimental repeat of each pUL37x1 over-expressing and control lines. (NS, non-significant, ***,p<0.001)

Figure 4-6

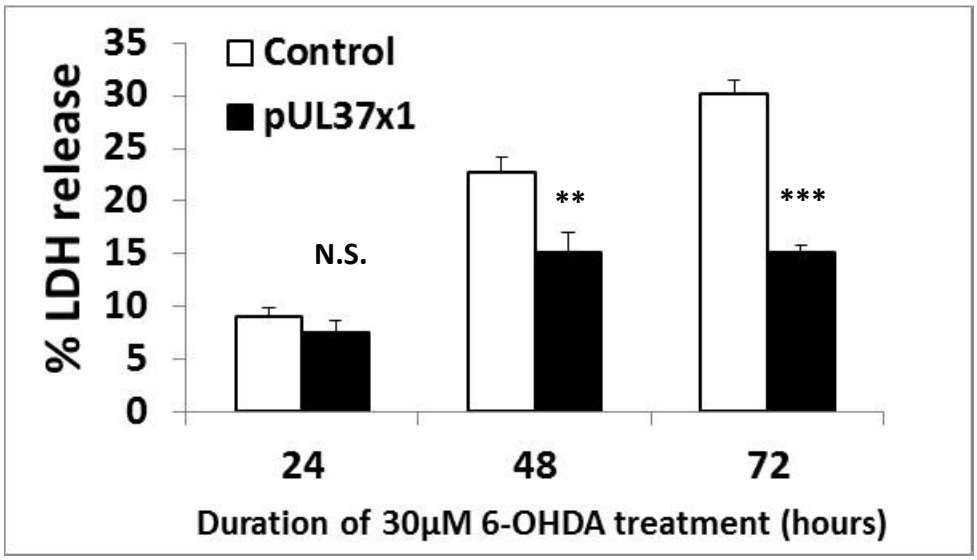

Figure 4-6. pUL37x1 over-expression significantly decreased the percentage of LDH release on SH-SY5Y cells induced by 30µM 6-OHDA treatment for 48 and 72 hours (48 hours: 22.8±1.5% in control, 15.1±1.9% in pUL37x1 over-expression, p<0.01, n=8; 72 hours: 30.2±1.3% in control, 15.1±0.6% in pUL37x1 over-expression, p<0.001, n=6). Data were presented as mean±S.E.M. Statistics was performed by two-tailed Student's t- test. The n numbers represented the experimental repeat of each pUL37x1 over-expressing and control lines (NS, non-significant, **,p<0.01; ***,p<0.001)

4.2.1.2 The Protection Was Investigated by PI Binding Assay

In order to confirm the protection of pUL37x1 over-expression, another cell death measurement was required. PI, a fluorescent protein which displays a marked increase its fluorescence when bound with nucleic acid, can only penetrate a cell membrane with impaired integrity. Two experimental conditions, which demonstrated the protection of pUL37x1 over-expression against 6-OHDA in the LDH release assay, were tested by PI binding assay to confirm the results. pUL37x1 over-expression provided a significant reduction of PI fluorescence in both 60µM 6-OHDA treatment for 24 hours and 30µM 6-OHDA treatment for 48 hours (60 µM 6-OHDA, 24hours: 33.6±1.5% in control, 22.2±2.0% in pUL37x1 over-expression, p<0.001, n=6; 30µM 6-OHDA, 48hours: 56.9±3.4% in control, 44.7±4.0% in pUL37x1

over-expression, p<0.05. n=6) (Figure 4-7). In this assay, dsRed-Mito cells were not included in the control because of the endogenous fluorescence they contain.

The protection conferred by pUL37x1 over-expression against 6-OHDA induced neuronal death is consistent as shown by both LDH release and PI binding assays.

Figure 4-7

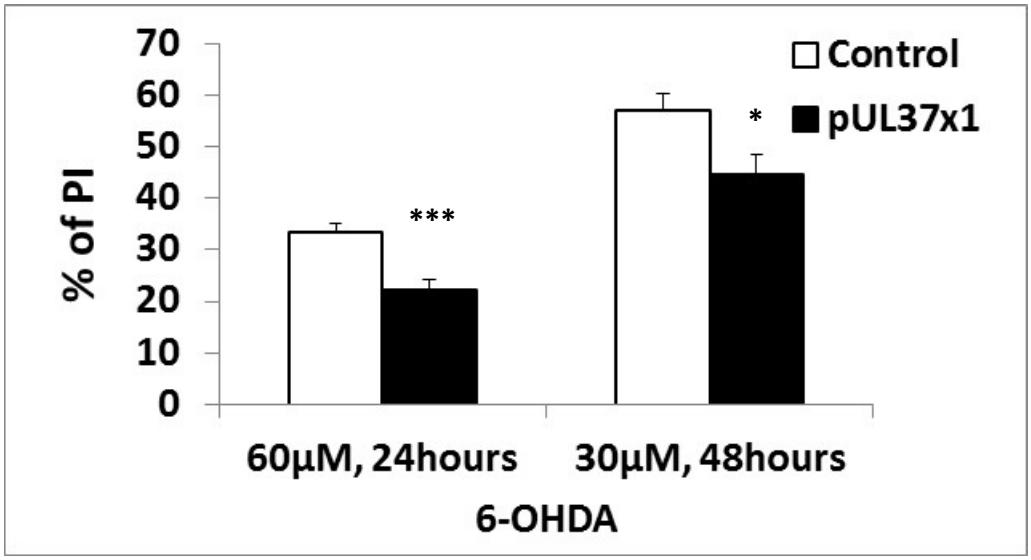

Figure 4-7. pUL37x1 over-expression significantly decreased the percentage of PI fluorescence on SH-SY5Y cells induced by 60 µM 6-OHDA treatment for 24hours and 30µM 6-OHDA treatment for 48hours (60 µM 6-OHDA, 24hours: 33.6±1.5% in control, 22.2±2.0% in pUL37x1 over-expression, p<0.001, n=6; 30µM 6-OHDA, 48hours: 56.9±3.4% in control, 44.7±4.0% in pUL37x1 over-expression, p<0.05. n=6). Data were presented as mean±S.E.M. Statistics was performed by two-tailed Student's t- test. The n numbers represented the experimental repeat from each pUL37x1 over-expression lines and control (normal SH-SY5Y cells and over-expressing pcDNA Zero SH-SY5Y cells) (*, p<0.05, **,p<0.001)

4.2.2 pUL37x1 over-expression Protected Staurosporine Induced Cytotoxicity

4.2.2.1 The Protection Was Investigated by LDH Release Assay

Staurosporine is able to induce substantial apoptotic cell death but pUL37x1 over-expression prevented this toxic effect. pUL37x1 over-expression significantly reduced the percentage of LDH release induced by 15nM staurosporine treatment

for either 24 or 48 hours treatment (24 hours: 25.4±2.3% in control, 13.9±0.8% in pUL37x1 over-expression, p<0.01, n=4; 48 hours: 22.8±1.5% in control, 15.1±1.9% in pUL37x1 over-expression, p<0.01, n=6) (Figure 4-8).

Furthermore, pUL37x1 over-expression significantly reduced the cell death induced by 30nM staurosporine treatment for 24 hours (22.3±1.3% in control, 13.7±0.5% in pUL37x1 over-expression, p<0.001, n=8) (Figure 4-9). Considering these results, pUL37x1 over-expression successfully demonstrates the ability to lower the apoptotic cell death induced by staurosporine. The dosage and duration of staurosporine was mainly based on the data of validation (Sec 2.5.1.3).

Figure 4-8

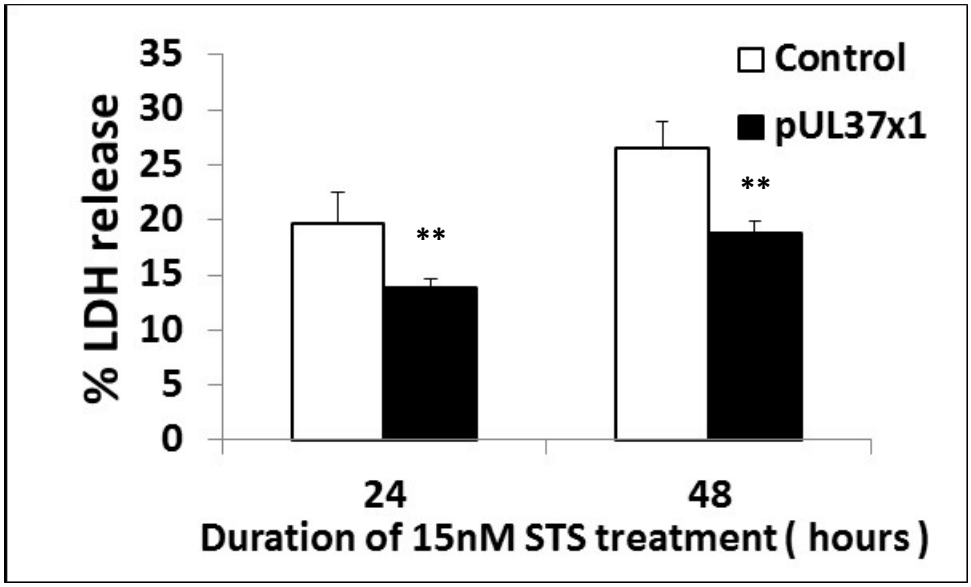

Figure 4-8. pUL37x1 over-expression significantly reduced the percentage of LDH release on SH-SY5Y cells induced by 15nM staurosporine treatment for 24 and 48 hours (24 hours: 25.4±2.3% in control, 13.9±0.8% in pUL37x1 over-expression, p<0.01, n=4; 48 hours: 22.8±1.5% in control, 15.1±1.9% in pUL37x1 over-expression, p<0.01, n=6). Data were presented as mean±S.E.M. Statistics was performed by two-tailed Student's t- test. The n number represented the experimental repeat from each pUL37x1 over-expression lines and control (normal SH-SY5Y cells, over-expressing pcDNA Zero SH-SY5Y cells and over-expressing dsRed-mito SH-SY5Y cells) (**,p<0.01).

Figure 4-9

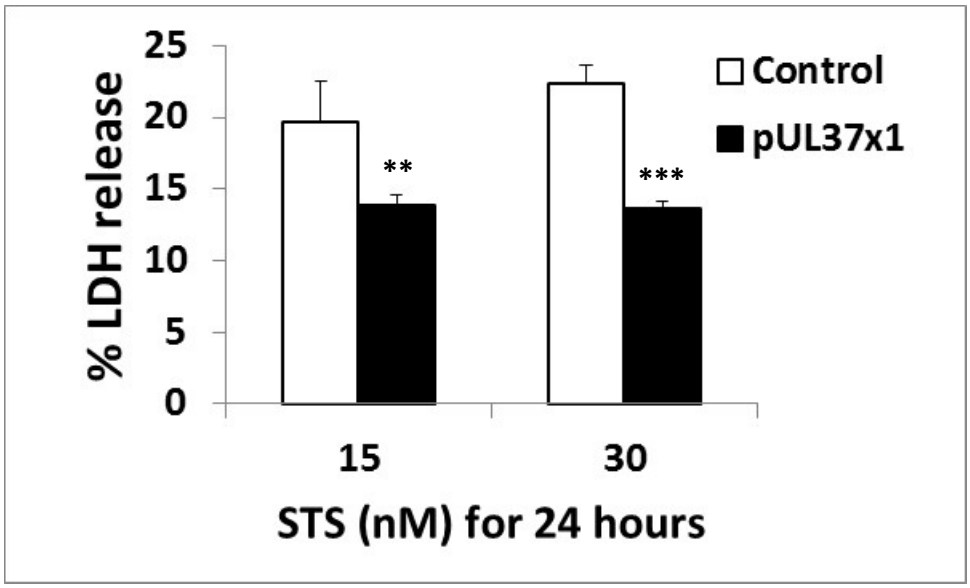

Figure 4-9. pUL37x1 over-expression significantly protected cell death on SH-SY5Y cells upon either 15 or 30nM staurosporine (STS) treatment for 24 hours (15nM: 25.4±2.3% in control, 13.9±0.8% in pUL37x1 over-expression, p<0.01, n=4; 30nM: 22.3±1.3% in control, 13.7±0.5% in pUL37x1 over-expression, p<0.001, n=8). Data were presented as mean±S.E.M. Statistics was performed by two-tailed Student's t- test. The n number represented the experimental repeat from each pUL37x1 over-expression lines and control (normal SH-SY5Y cells, over-expressing pcDNA Zero SH-SY5Y cells and over-expressing dsRed-mito SH-SY5Y cells) (**,p<0.01, ***, p<0.001).

4.2.2.2 pUL37x1 Protection Was Investigated by PI Binding Assay

The protection of pUL37x1 over-expression against staurosporine was also confirmed by PI binding assay. Illustrated by figure 4-10, pUL37x1 over-expression significantly lowered the PI fluorescence induced by either 15nM staurosporine treatment for 48 hours or 30nM staurosporine treatment for 24 hours (15nM treatment for 48 hours: 22.9±1.4% in control, 14.7±1.5% in pUL37x1 over-expression, p<0.001, n=8; 30nM treatment for 24 hours: 12.5±2.4% in control, 6.8±1.3% in pUL37x1 over-expression, p<0.05, n=6). These results confirm the protection of pUL37x1 over-expression from staurosporine-induced cytotoxicity.

Figure 4-10

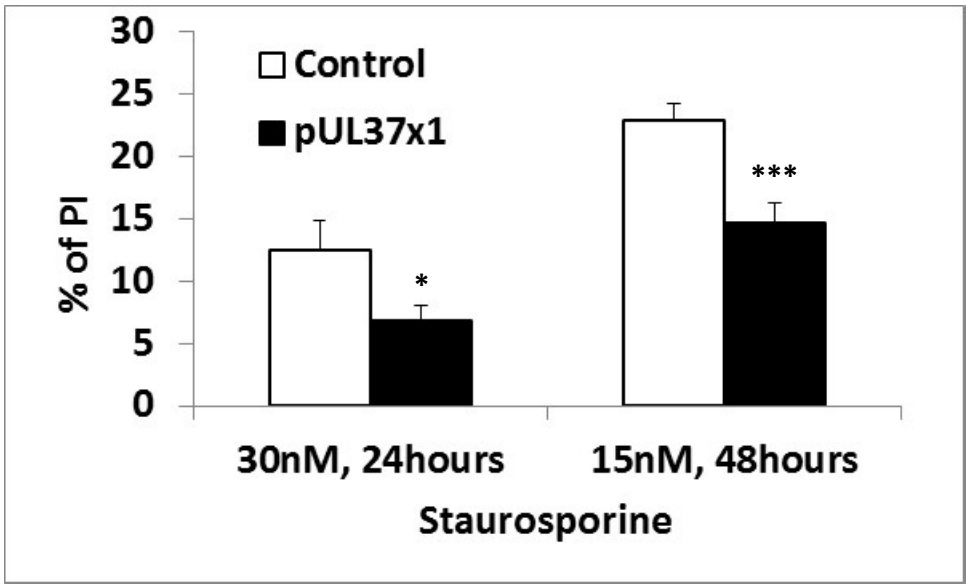

Figure 4-10. pUL37x1 over-expression reduced the PI fluorescence on SH-SY5Y cells induced by staurosporine. The protection was noted in either 15nM treatment for 48 hours or 30nM treatment for 24 hours (15nM treatment for 48 hours: 22.9±1.4% in control, 14.7±1.5% in pUL37x1 over-expression, $p<0.001$, n=8; 30nM treatment for 24 hours: 12.5±2.4% in control, 6.8±1.3% in pUL37x1 over-expression, $p<0.05$, n=6). Data were presented as mean±S.E.M. Statistics was performed by two-tailed Student's t- test. The n number represented the experimental repeat from each pUL37x1 over-expression lines and control (normal SH-SY5Y cells, and over-expressing pcDNA Zero SH-SY5Y cells) (*,$p<0.05$, ***, $p<0.001$).

4.3 pUL37x1 Over-expression Modulated Mitochondria-dependent Apoptosis

As mentioned in the introduction, pUL37x1 modulates mitochondria-dependent apoptosis. Several steps within mitochondria-dependent apoptosis were evaluated on pUL37x1 over-expressing SH-SY5Y cells, including Bax translocalization, change of mitochondrial membrane potential, release of cytochrome c and activation of caspase-3, to test this hypothesis and possibly identify the mechanism(s) of anti-apoptotic action.

4.3.1 pUL37x1 Over-expression Induced Mitochondria-localization of Bax

Bax is one of the pro-apoptotic proteins in Bcl-2 family. In the absence of toxin, most Bax protein is in the cytoplasm, however, upon apoptosis, it translocates to mitochondria and dimerises. The dimer is inserted into the mitochondrial outer membrane and induces MMP, which results in the release of inner mitochondrial contents and causes cell death. It has been reported that pUL37x1 leads to the translocation of Bax from cytoplasm to mitochondria during CMV infection. However, instead of activating apoptosis, Bax proteins were inactivated after translocation. This process is thought to be responsible for the anti-apoptotic mechanism of pUL37x1.

In terms of the pUL37x1 over-expressing SH-SY5Y cells, the translocation of Bax was also clearly demonstrated by immunocytochemistry investigation. Cells were co-stained with MitoTracker red, a mitochondrial dye and anti-Bax antibody (green). Images were obtained by confocal microscope. Control SH-SY5Y cells revealed a homogenous intracellular distribution of Bax without obvious co-localization with MitoTracker red. In contrast, pUL37x1 over-expressing cells exhibited a remarkable translocation of Bax: there were numerous co-localization of Bax with MitoTracker red, which indicated a mitochondrial translocation of Bax (Figure 4-11).

Figure 4-11

Control pUL37x1 o/e

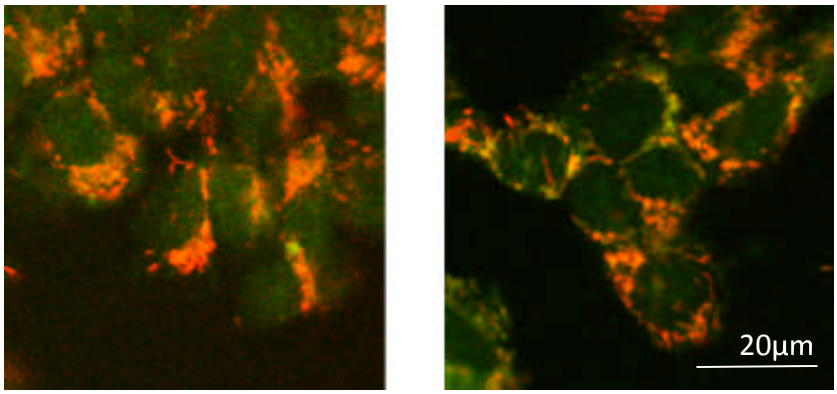

Figure 4-11. pUL37x1 over-expression affected the intra-cellular localization of Bax. In control SH-SY5Y cells, Bax (green) demonstrated a predominant intracellular distribution with scant co-localization with MitoTracker (red). Nevertheless, in pUL37x1 over-expressing (pUL37x1 o/e) SH-SY5Y cells, a remarkable co-localization with Bax and MitoTracker was shown (yellow), which indicated a translocation of Bax from cytoplasm to mitochondria. Images were obtained from confocal microscope.

4.3.2 pUL37x1 Over-expression Hyperpolarized Mitochondria and Rescued 6-OHDA Induced Depolarization

Depolarization of mitochondria is a common phenomenon during apoptosis. It has been reported that mitochondrial depolarization takes place preceding Bax translocation(Smaili et al., 2001). Also, hyperpolarized mitochondria have been shown as an anti-apoptosis strategy in the meclizine section of this study. Considering the importance of mitochondrial membrane potential in apoptosis, it was worth investigating whether pUL37x1 over-expression affected it or not.

In the absence of toxin, pUL37x1 over-expressing SH-SY5Y cells exhibited hyperpolarized mitochondria compared with control SH-SY5Y cells (control: 100.0±1.4%, UL37: 175.3±7.4%,p<0.001). This hyperpolarization prevented the depolarization induced by 100μM 6-OHDA treatment for 1 hour (control: 77.0±2.8%, UL37: 114.1±3.2%,p<0.001) (Figure 4-12). 100μM 6-OHDA treatment on SH-SY5Y

cells had been proved to induce mitochondrial depolarization 1 hour after treatment(Lotharius et al., 1999). TMRM fluorescence from each experiment was normalized by the average of TMRM fluorescence obtained from no-toxin SH-SY5Y cells. Therefore, pUL37x1 over-expression results in mitochondrial hyperpolarization in SH-SY5Y cells and protects the apoptosis-triggered depolarization.

Figure 4-12

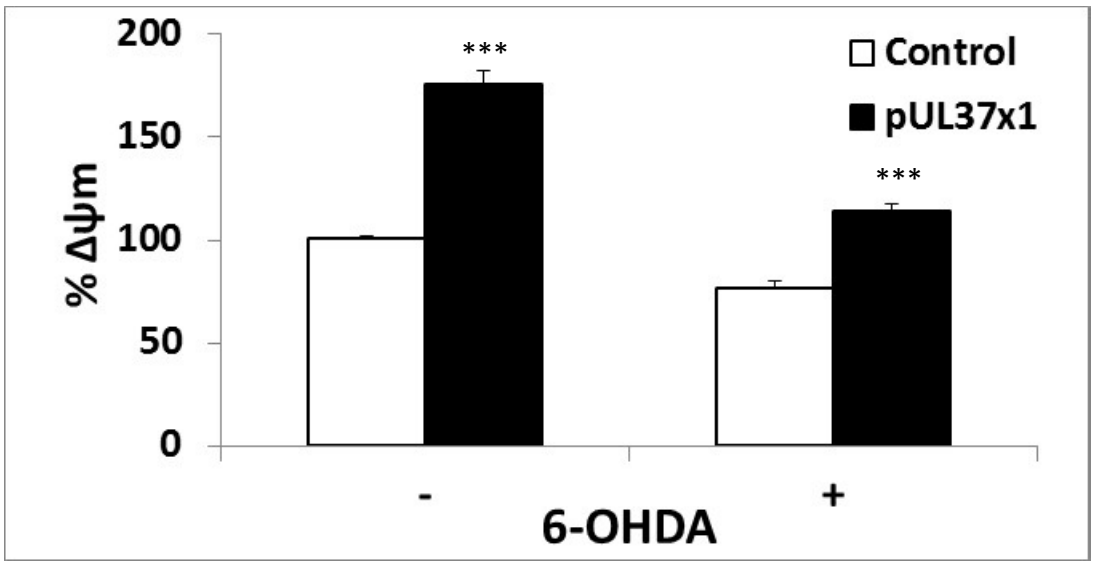

Fig 4-12. pUL37x1 over-expression significantly hyperpolarized baseline mitochondrial membrane potential on SH-SY5Y cells (175.3±7.4% compared with 100.0±1.4% from control, p<0.001, n=24 from control SH-SY5Y cells and n=8 from each pUL37x1 over-expressing line) and rescued the mitochondrial depolarization induced by 100μM 6-OHDA treatment for 1 hour (114.1±3.2% compared with 77.0±2.4% from control, p<0.001, n=10 from control SH-SY5Y cells and n=5 from each pUL37x1 over-expressing line). Mitochondrial membrane potential was measured by TMRM fluorescence and normalized by the average of TMRM fluorescence obtained from no-toxin SH-SY5Y cells. Data were presented as mean±S.E.M. Statistics was performed by two-tailed Student's t- test. (***, p<0.001)

4.3.3 pUL37x1 Over-expression Reduced Cytochrome c Release to Cytoplasm

Mitochondrial contents are released into cytoplasm after MMP and some of these result in the activation of further apoptotic cascades. Cytochrome c, an electron

carrier between complex III and IV, is also well-known to induce apoptosis when it is released into the cytoplasm. pUL37x1 over-expression significantly prevents the release of cytochrome c from mitochondria to cytoplasm. When no toxin is present, the cytosolic concentration of cytochrome c was lower in the pUL37x1 over-expression cells compared with control (control: 0.29±0.04, pUL37x1: 0.17±0.02 ng/mg cell lysate,p<0.05). Following 500nM staurosporine treatment for 3 hours, cytochrome c concentration surged in the control whereas pUL37x1 over-expressing cells significantly blocked the release (control: 0.46±0.05, pUL37x1: 0.21±0.03 ng/mg cell lysate,p<0.01) (Fig 4-13). The dosage and duration of staurosporine is based on previous study (McGinnis et al., 1999). Based on these findings, pUL37x1 over-expression is able to block the release of mitochondrial apoptotic contents, such as cytochrome c into cytoplasm and avoid the further activation of apoptotic cascades.

Figure 4-13

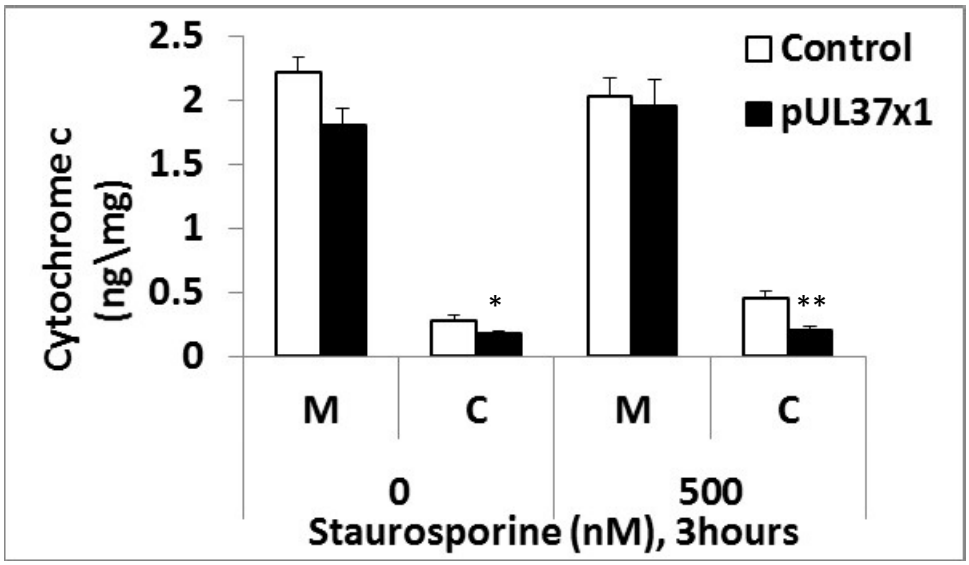

Figure 4-13. pUL37x1 over-expression did not alter the baseline cytochrome c level in mitochondrial fraction (M) of cell lysate on SH-SY5Y cells. However, it significantly reduced cytochrome c releasing to cytoplasm (C) in no-toxin status and upon 500nM staurosporine treated for 3 hours (no-toxin: 0.29±0.04 ng/mg cell lysate in control versus 0.17±0.02

ng/mg cell lysate in pUL37x1 over-expression lines,p<0.05, n=3; 500nM staurpsoprone: 0.46±0.05 ng/mg cell lysate in control versus 0.21±0.03 ng/mg cell lysate in pUL37x1 over-expression lines,p<0.01, n=3). Data were presented as mean±S.E.M . The n number represented the experimental repeat from each pUL37x1 over-expression lines and control (include normal SH-SY5Y cells, over-expressing dsRed-mito SH-SY5Y cells and over-expressing pcDNA Zero SH-SY5Y cells). Statistics was performed by two-tailed Student's t-test. (*,p<0.05, **, p<0.01)

4.3.4 pUL37x1 Over-expression Reduced Staurosporine Induced Activation of Caspase-3

The activation of caspase-3 is at the late stage of apoptosis and caspase-3 activity is recognized as a parameter of apoptosis. pUL37x1 over-expression was expected to prevent the activation of caspase-3 due to blocking of the upstream apoptosis process.

In the absence of toxin, pUL37x1 over-expression cells did not alter the activity of caspase-3 compared with control (control: 112.6±6.3, pUL37x1: 111.9±6.0%, p>0.05). However, following 500nM staurosporine treatment for 4 hours, pUL37x1 over-expressing SH-SY5Y cells exhibited significantly lower caspase-3 activity compared with control (control: 1156.5±137.2, pUL37x1: 440.5±56.3%, p<0.001) (Figure 4-14). The caspase-3 activity was normalized by using the average of activity pfrom no-toxin SH-SY5Y cells. The dosage and duration of staurosporine treatment on SH-SY5Y cells was based on previous report from McGinnis et al(McGinnis et al., 1999).

In conclusion, pUL37x1 over-expression confirms the hypothesis of modulation of mitochondria-dependent apoptosis. It leads to mitochondrial translocalization of Bax without initiation of apoptosis. In addition, this anti-apoptotic protein hyperpolarizes mitochondria and prevents their depolarization during apoptosis.

These phenomenon result in less mitochondrial cytochrome c release and lower caspase-3 activity following apoptotic stress induction.

Figure 4-14

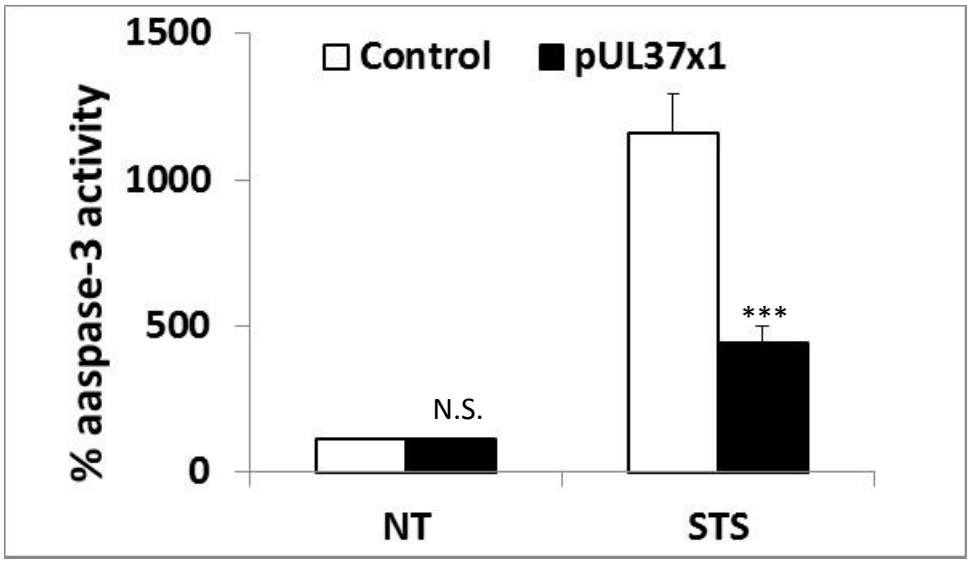

Fig 4-14. pUL37x1 over-expression did not alter the baseline caspase-3 activity on SH-SY5Y cells but significantly lowered the increase of caspase-3 activity upon 500nM staurosporine treated for 4 hours (1156.5±137.2% in control versus 440.5±56.3% in pUL37x1 over-expression, p<0.001, n=5, data were normalized by averaged caspase-3 activity from the no-toxin SH-SY5Y cells). Data were presented as mean±S.E.M.. The n numbers represented the experimental repeat from each pUL37x1 over-expressing lines and control (include normal SH-SY5Y cells and over-expressing pcDNA Zero SH-SY5Y cells). Statistics was performed by two-tailed Student's t- test. (NS, non-significant, ***,p<0.001)

4.4 The Protection of pUL37x1 Over-expression Was due to Bax Inactivation

In section 4.3.1, pUL37x1 over-expression caused mitochondrial translocalization of Bax without initiation of apoptosis. pUL37x1 is believed to inactivate Bax after the translocalization. Provided that the inactivation of Bax occurs, pUL37x1 over-expressing cells would not obtain more protection from Bax ablation, which has been reported to reduce apoptosis and cell death.

Both control and pUL37x1 over-expressing cells were treated with either scramble siRNA and Bax siRNA for 3 days. The protein level of Bax was reduced to less than

20% following Bax siRNA silencing compared with scramble siRNA in both control and pUL37x1 over-expressing cells (Figure 4-15). Challenging with 30nM staurosporine for 24 hours, Bax siRNA silencing reduced the percentage of LDH release compared with scramble siRNA silencing in control cells (Scr siRNA: 20.9±1.4, Bax siRNA: 16.2±1.2%, p<0.01). Under scramble siRNA silencing, pUL37x1 over-expression remained protective against staurosporine compared with same condition control (control: 20.9±1.4, pUL37x1: 12.7±0.9%, p<0.001). However, Bax siRNA silencing failed to produce more protection on pUL37x1 over-expressing cells, there was no difference of cell death between control and pUL37x1 over-expressing cells (control: 16.2±1.2, pUL37x1: 15.4±1.5%, p>0.05) (Figure 4-16).

These data indicate that the anti-apoptotic effect of pUL37x1 over-expression is due to Bax inactivation and Bax siRNA silencing fails to produce more protection, in contrast to the control cells.

Figure 4-15

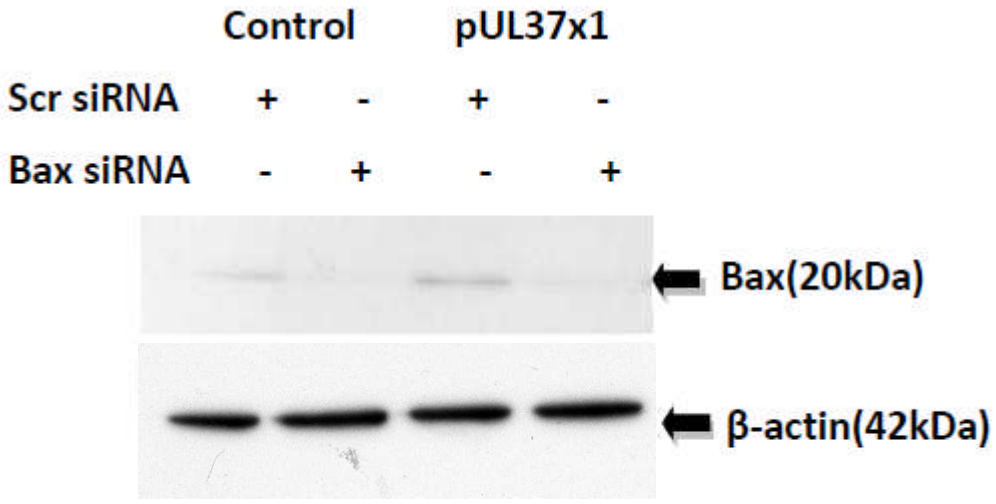

Figure 4-15. Representative western blot analysis image demonstrated that 5nM Bax siRNA silencing for 3 days remarkably lowered the Bax protein level in both control and pUL37x1 over-expressing SH-SY5Y cells compared with scramble siRNA (Scr) silencing.

Figure 4-16

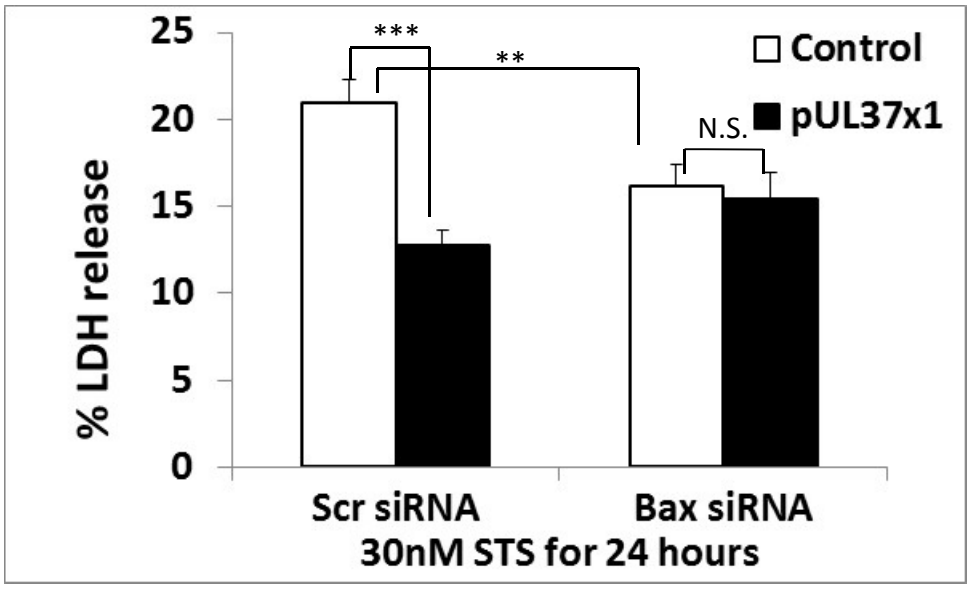

Figure 4-16. 5nM Bax siRNA silencing for 3 days significant reduced LDH release on SH-SY5Y cells upon 30nM staurosporine treated for 24 hours (20.94±1.35% in scramble siRNA versus 16.19±1.09% in Bax siNRA, $p<0.01$, n=8). However, this reduction did not present in pUL37x1 over-expressing SH-SY5Y cell. In addition, there was no significant difference of LDH release upon 30nM staurosporine treated for 24 hours between controls with pUL37x1 over-expressing SH-SY5Y cells when both of them underwent 5nM Bax siRNA silencing. Data were presented as mean±S.E.M. Statistics was performed by two-tailed Student's t-test. The n number represented the experimental repeat from each pUL37x1 over-expression lines and control (include normal SH-SY5Y cells, over-expressing dsRed-mito SH-SY5Y cells and over-expressing pcDNA Zero SH-SY5Y cells) (NS, non-significant, **,$p<0.01$; ***,$p<0.001$)

4.5 The protection of pUL37x1 Over-expression is mediated by Mitochondrial Hyperpolarization and Glycolysis

It has been demonstrated in section 4.3.2 that pUL37x1 over-expression results in mitochondrial hyperpolarization at both basal and 6-OHDA treated status. It has also been proved, in previous literature and present studies with meclizine, that avoiding mitochondrial depolarization is a protective mechanism . This suggests that, as well as Bax inactivation, there is another anti-apoptotic effect of pUL37x1.

It was suspected that pUL37x1 induced mitochondrial hyperpolarization through increased glycolysis by two factors. First, as described section 1.5.3.2,

hyperpolarization can result from complex V inhibition, insufficient ADP supply or increased glycolysis. However, the two former would impair the ATP synthesis and lead to energy deficit and because pUL37x1 over-expression does not result in increased cell death, it is unlikely that the viral protein causes a shortage of ATP. Second, CMV infection has been found to increase the glucose metabolism in host cells, which may be associated with pUL37x1.

4.5.1 Mitochondrial Uncoupling Attenuated the Protection

CCCP was applied to test the mitochondrial hyperpolarization-dependent protection of pUL37x1 over-expression. Figure 4-17 demonstrated that 1μM CCCP was able to depolarize either control or pUL37x1 over-expressing SH-SY5Y cells without significant difference (34.3±2.3% compared with 33.0±2.3% from control, p>0.05) .

Figure 4-17

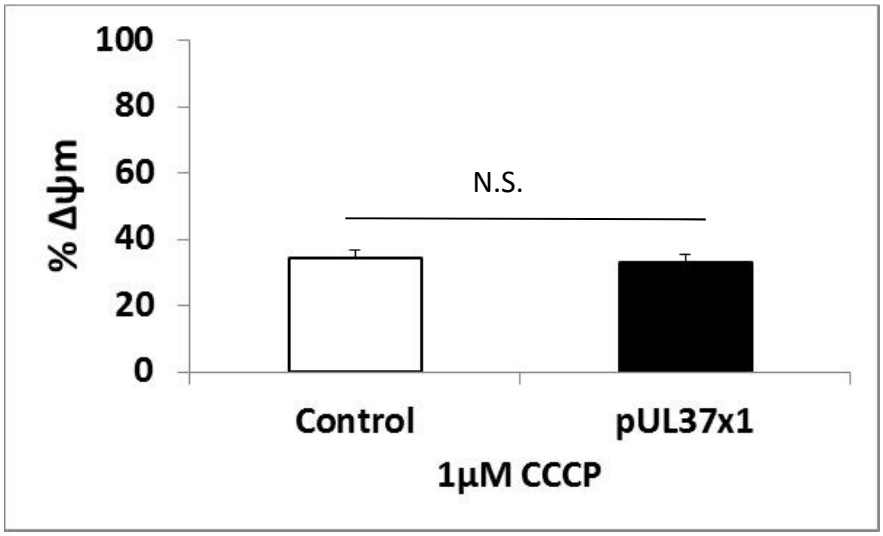

Figure 4-17. 1μM CCCP depolarized mitochondria in either control or pUL37x1 over-expressing SH-SY5Y cells (34.3±2.3% compared with 33.0±2.3% from control, p>0.05, n=6). TMRM fluorescence from each experiment was normalized by the average of TMRM fluorescence obtained from no-toxin SH-SY5Y cells. Data were presented as mean±S.E.M . The n number represented the experimental repeat from each pUL37x1 over-expressing

lines and control SH-SY5Y cells. Statistics was performed by two-tailed Student's t- test. (N.S., non-significant)

Since CCCP depolarized pUL37x1 over-expressing SH-SY5Y and control cells, it was applied to test cell death protection. 1μM CCCP treatment for 48 hours resulted in 22.7±2.5% of LDH release in control SH-SY5Y compared to 20.8±2.1% in pUL37x1 over-expressing cells (Figure 4-18). This result suggests that there was no protection conferred by pUL37x1 over-expression in the absence of mitochondrial hyperpolarization effect.

Figure 4-18

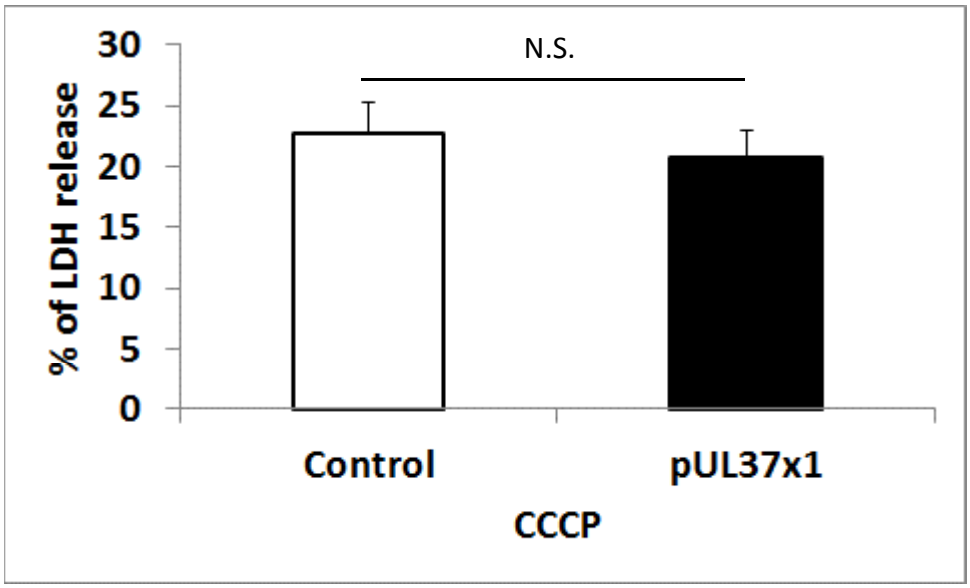

Figure 4-18. pUL37x1 over-expression failed to protect cell death upon 1μM CCCP treatment for 48 hours (control: in 22.7±2.5%; pUL37x1 over-expression: 20.8±2.1%, p>0.05, n=8). The n number represented the experimental repeat from each pUL37x1 over-expressing lines and control (include normal SH-SY5Y cells, over-expressing dsRed-mito SH-SY5Y cells and over-expressing pcDNA Zero SH-SY5Y cells). Statistics was performed by two-tailed Student's t- test. (N.S., non-significant)

4.5.2 pUL37x1 Over-expression Increased the Glycolytic Activity without Altering OXPHOS and ADP Phosphorylation Activity

pUL37x1 over-expressing and control SH-SY5Y cells were cultured in a microplate for XF analysis. Glycolysis was measured by ECAR and OXPHOS was measured by OCR. pUL37x1 over-expression significantly increased the ECAR compared with control (control: 1.39±0.20, pUL37x1: 2.68±0.46 pH/min/µg, $p<0.05$). However, this glycolysis-enhancing effect did not result from OXPHOS inhibition as there was no significant difference in OCR between control with pUL37x1 over-expressing SH-SY5Y cells (control: 5.69±1.34, pUL37x1: 6.29±1.73 nmole/min/µg, $p>0.05$) (Figure 4-19).

pUL37x1 over-expression affected neither general OXPHOS activity nor the ATP synthesis capability from each respiratory chain complex. Assessing by ADP phosphorylation assay, there was no difference between pUL37x1 over-expression and control SH-SY5Y cells in ATP synthesis capability from each complex (control: complex I+II+III+IV: 26.3±3.6, complex II+III+IV: 19.3±2.0, complex IV: 2.7±0.9; pUL37x1: complex I+II+III+IV: 28.9±2.5, complex II+III+IV: 13.9±1.7, complex IV: 4.6±0.6 pmole ATP/min/mg, $p>0.05$ in each condition) (Figure 4-20).

These results revealed that pUL37x1 over-expression causes the increased glycolysis in SH-SY5Y cells without impairing OXPHOS. This finding supports the speculation that pUL37x1 leads to mitochondrial hyperpolarization due to enhancing glycolysis.

Figure 4-19

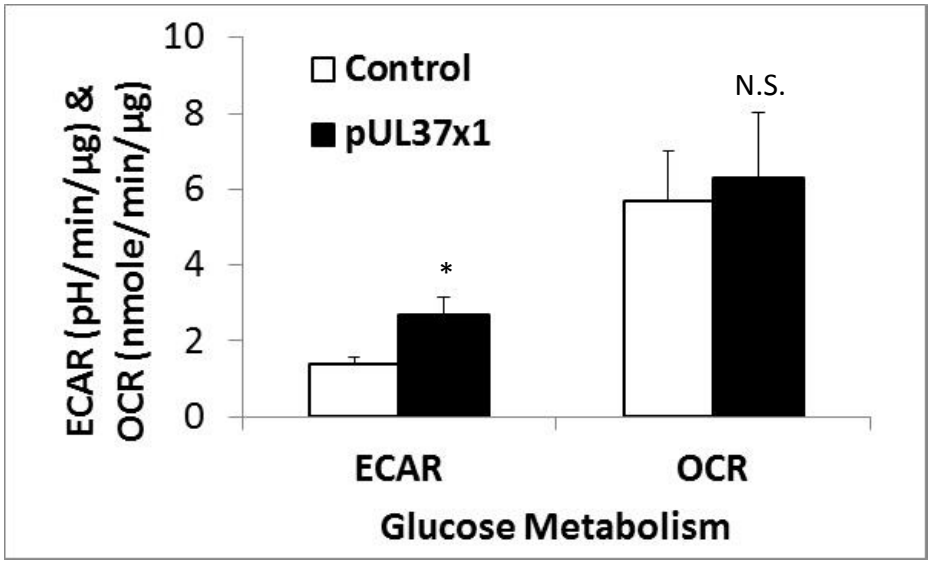

Figure 4-19. Glycolysis and OXPHOS of SH-SY5Y cells were measured by XF analyser. pUL37x1 over-expression induced a significant increase of ECAR compared with control (pUL37x1: 2.68±0.46, control: 1.39±0.20 pH/min/µg, p<0.05, n=10) without impaired OXPHOS as measure by OCR. Data were presented as mean±S.E.M. Statistics was performed by two-tailed Student's t- test. The n number represented the experimental repeat from each pUL37x1 over-expressing lines and control (include normal SH-SY5Y cells, over-expressing dsRed-mito SH-SY5Y cells and over-expressing pcDNA Zero SH-SY5Y cells). (NS, non-significant, *,p<0.05).

Figure 4-20

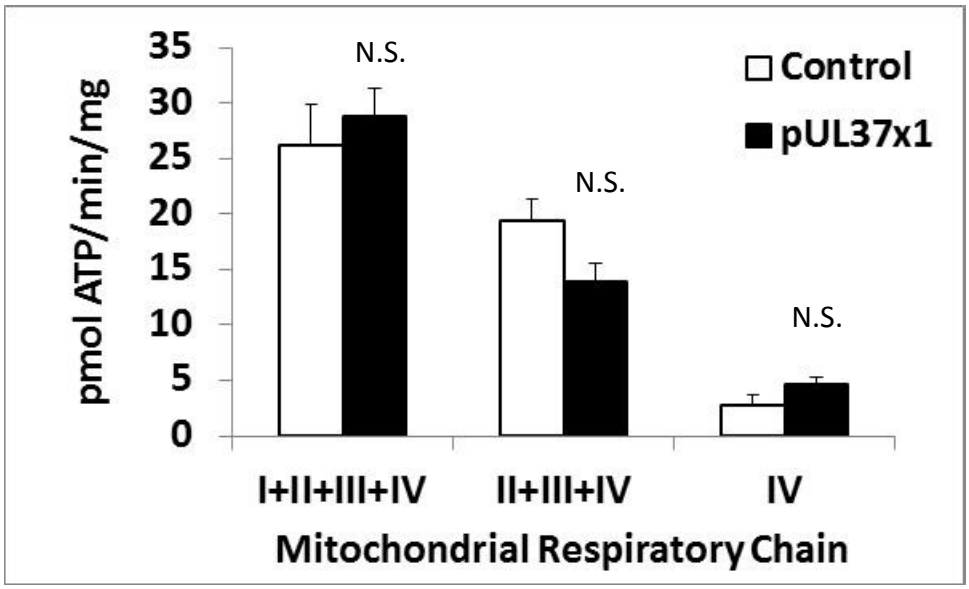

Figure 4-20. pUL37x1 over-expression did not affect mitochondrial ADP phosphorylation ability from each respiratory chain complex on SH-SY5Y cells (control: complex I+II+III+IV: 26.3±3.6, complex II+III+IV: 19.3±2.0, complex IV: 2.7±0.9; pUL37x1: complex I+II+III+IV: 28.9±2.5, complex II+III+IV: 13.9±1.7, complex IV: 4.6±0.6 pmole ATP/min/mg, p>0.05 in

each condition, n=3). The n number represented the experimental repeat from each pUL37x1 over-expressing lines and control SH-SY5Y cells. (NS, non-significant).

4.5.3 pUL37x1 Over-expression Induced Mitochondrial Hyperpolarization Was Glycolysis-dependent

Glycolytic inhibitors (2DG and 3BP) were applied to pUL37x1 over-expressing SH-SY5Y cells 48 hours before measuring mitochondrial membrane potential to verify glycolysis-dependent mitochondrial hyperpolarization. In control SH-SY5Y cells, neither 10μM 2DG nor 5μM 3BP altered the mitochondrial membrane potential (control: 100.0±2.3, 2DG: 97.0±4.4, 3BP: 97.6±2.3%, TMRM fluorescence was adjusted by mitochondrial content and normalized by the average TMRM fluorescence of control SH-SY5Y cells, $p>0.05$). However, the hyperpolarization induced by pUL37x1 over-expression disappeared following treatment with either 2DG or 3BP (pUL37x1: 163.6±9.3, pUL37x1+2DG: 103.7±1.9, 3BP: 87.8±2.8%, $p<0.001$ in both conditions, TMRM fluorescence was adjusted by mitochondrial content and normalized by the average TMRM fluorescence of control SH-SY5Y cells) (Figure 4-21).

This result confirms that the hyperpolarization does depend on the glycolysis-enhancing effect of pUL37x1 over-expression.

Figure 4-21

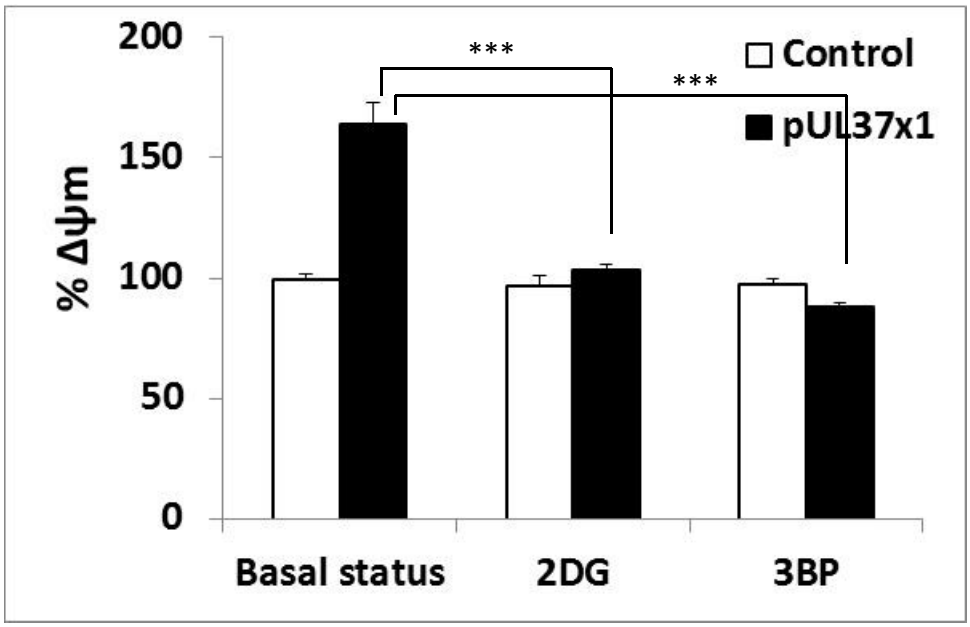

Figure 4-21. Glycolytic inhibitors, either 10μM 2-deoxy-glucose (2DG) or 5μM 3-Bromopyruvate (3BP) treatment for 48 hours, significantly reversed the phenomenon of mitochondrial hyperpolarization resulted from pUL37x1 over-expression (pUL37x1: 163.6±9.3%, pUL37x1+10μM 2DG: 103.7±1.9%, pUL37x1+5μM 3BP: 87.8±2.8%, p<0.001 respectively, n=10). The TMRM fluorescence was adjusted by mitochondrial content and normalized by the average TMRM fluorescence of control SH-SY5Y cells. Data were presented as mean±S.E.M. Statistics was performed by one-ANOVA with Dunnett's post-hoc analysis. The n number represented the experimental repeat from each pUL37x1 over-expression lines and control SH-SY5Y cells (***, p<0.001).

4.5.4 The Protection of pUL37x1 Over-expression against 6-OHDA Was Glycolysis-dependent

Providing the mitochondrial hyperpolarization and anti-apoptosis of pUL37x1 over-expression is glycolysis-dependent, glycolysis inhibition should, not only attenuate the potential difference, but also impair the protection. When control cells were challenged by 30μM 6-OHDA for 48 hours, co-administration of either 10μM 2DG or 5μM 3BP did not affect the percentage of LDH release (control: 24.1±1.8, 2DG: 28.2±1.9, 3BP: 25.8±2.2%, p>0.05). In contrast, applying these two glycolytic inhibitors simultaneously with 6-OHDA on pUL37x1 over-expressing cells

caused significant attenuation of protection (pUL37x1: 11.7±1.2, pUL37x1+2DG: 17.8±1.3, pUL37x1+3BP: 18.0±1.6%, p<0.01 in both conditions)(Figure 4-22).

In conclusion, mitochondrial hyperpolarization is another anti-apoptotic mechanism of pUL37x1. This phenomenon results from increasing glycolysis and disappears in the presence of glycolytic inhibitors. Without hyperpolarization, the protection against 6-OHDA cytotoxicity is lessened. These findings demonstrate that the protection and anti-apoptotic effects of pUL37x1 were glycolysis-dependent.

Figure 4-22

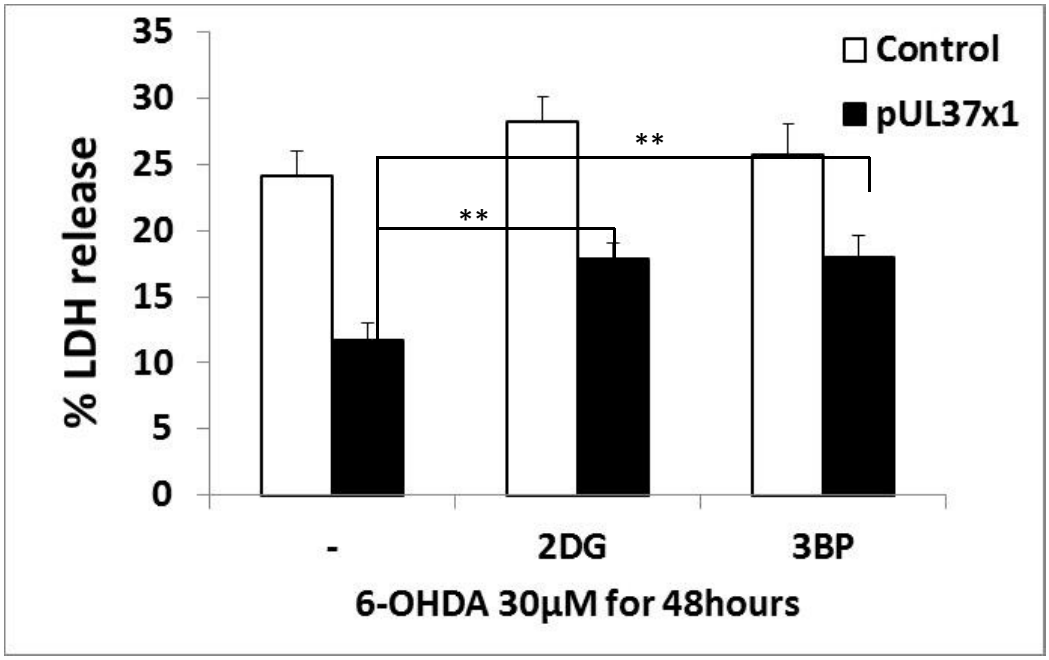

Figure 4-22. The protection of pUL37x1 over-expression against 30µM 6-OHDA, 48 hours treatment significantly attenuated while co-administering with either 10µM 2DG or 5µM 3BP (pUL37x1: 11.7±1.2%, pUL37x1+10µM 2DG: 17.8±1.3%, pUL37x1+5µM 3BP: 18.0±1.6%, p<0.01 respectively, n=6). Data were presented as mean±S.E.M. Statistics was performed by one-ANOVA with Dunnett's post-hoc analysis. The n number represented the experimental repeat from each pUL37x1 over-expressing lines and control (include normal SH-SY5Y cells, over-expressing dsRed-mito SH-SY5Y cells and over-expressing pcDNA Zero SH-SY5Y cells)(**, p<0.01).

4.6 pUL37x1 Over-expression Did Not Alter Glycolytic Enzymes Expression

It remains unclear how pUL37x1 up-regulates glycolysis. Increased expression of key glycolytic enzymes in host cells have been noted during CMV infection. However, there is no significant difference in these protein expression levels between control and pUL37x1 over-expressing cells (representative western blot analysis: Figure 4-23, densitometry analysis: Figure 4-24). pUL37x1 may, therefore, regulate glucose metabolism in other ways, such as the cellular glucose uptake or some post-translational modification of these enzymes.

Figure 4-23

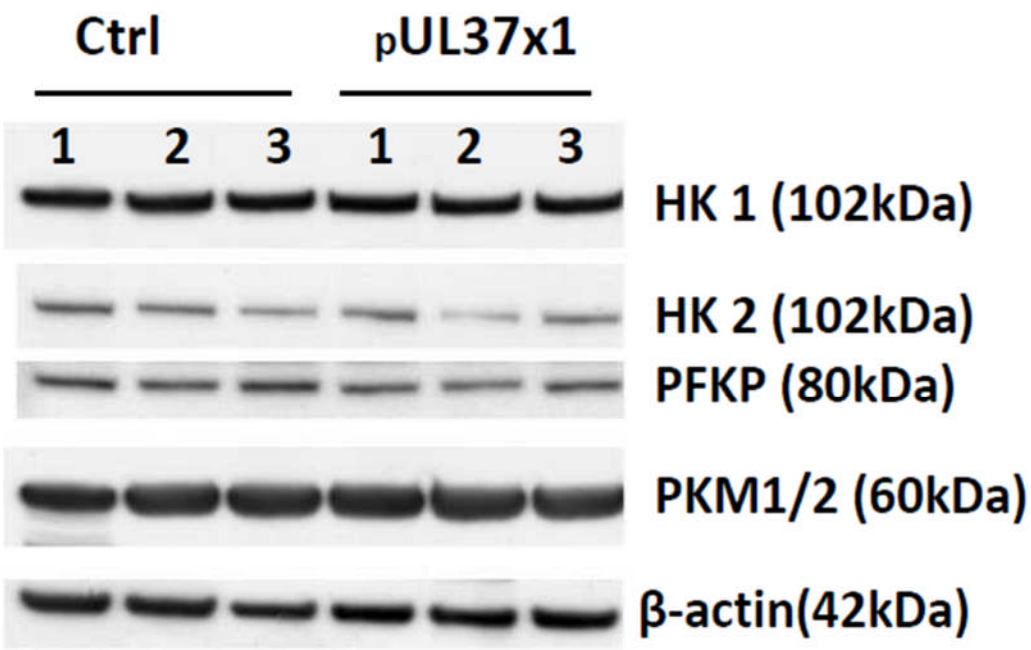

Figure 4-24

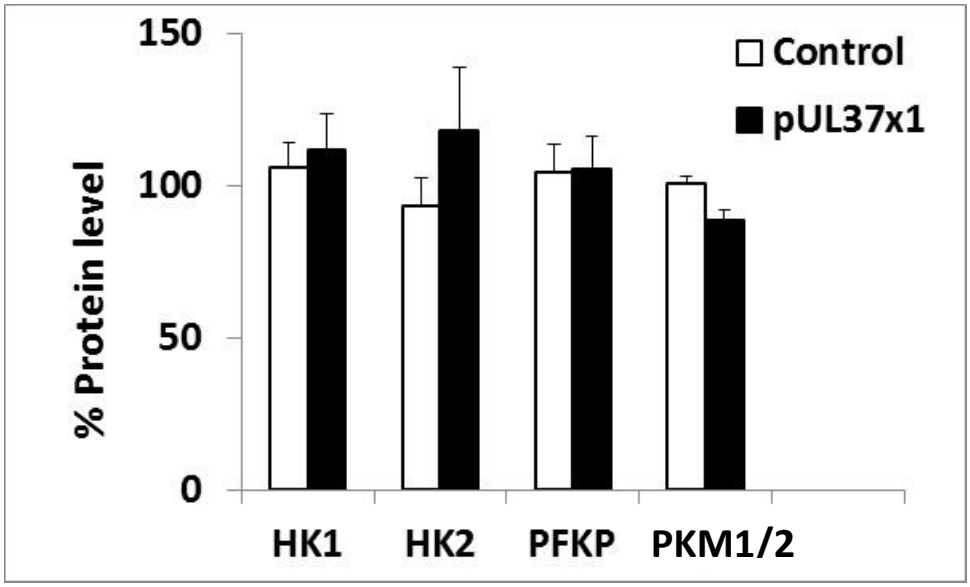

Figure 4-23 and 4-24. Representative western blot and densitometry analysis revealed that pUL37x1 over-expression did not alter the protein level of certain key glycolytic enzymes, including hexokinase 1 (HK1), hexokinase 2 (HK2), phosphofructokinase, platelet (PFKP) and pyruvate kinase isozymes M1/M2 (PKM1/2) on SH-SY5Y cells. The expression level was corrected by the level of β-actin, which served as a loading control (n=3). Data were presented as mean±S.E.M. Statistics was performed by two-tailed Student's t- test. . The n number represented the experimental repeat from each pUL37x1 over-expressing lines and control (include normal SH-SY5Y cells, over-expressing dsRed-mito SH-SY5Y cells and over-expressing pcDNA Zero SH-SY5Y cells).

4.7 pUL37x1 Over-expression Protected Apoptosis and Neuronal Death in Rat Primary Cortical Culture

Since pUL37x1 over-expression successfully demonstrated protection against toxin-induced apoptosis and cell death in the SH-SY5Y cell model. Next, primary rat cortical culture was utilized to further test the protection conferred by pUL37x1 over-expression. Liposomal transfecting reagent mixed with control red fluorescence protein (RFP) plasmid, a kind gift from Dr Guillaume Charras in London Centre for Nanotechnology, or a mixture of pUL37x1 and RFP plasmids at 10 to 1 ratio were co-incubated with culturing media for 48 hours. In control groups, cells with red fluorescence were recognized as RFP over-expressing cells. In pUL37x1

group, cells with red fluorescence were assumed to over-expressing both pUL37x1 and RFP because of the 10 times difference of the plasmid, which indicates that the cells expressed RFP have a very high possibility of over-expressing pUL37x1 as well. The percentage of successful transfection was about 5% and the transfection reagent did not cause significant cell death (Fig 4-25).

Figure 4-25

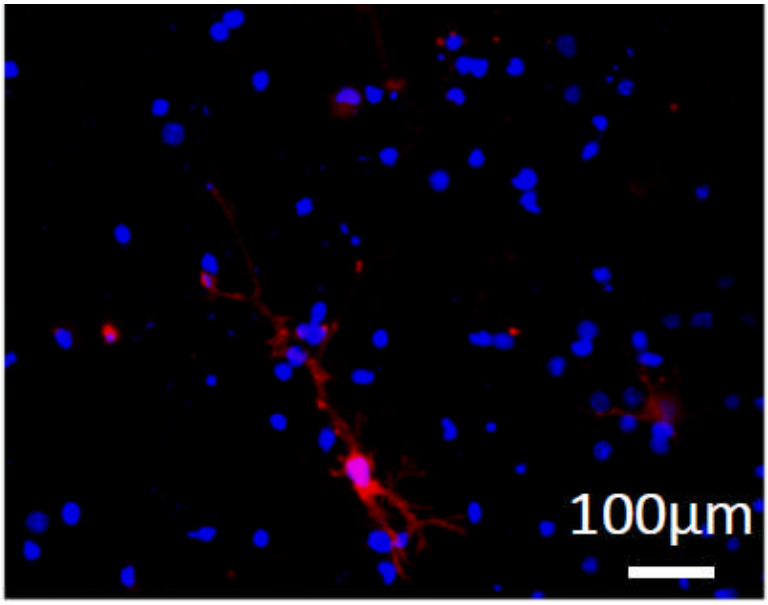

Figure 4-25. Mixture of pUL37x1 plasmid and red fluorescence protein (RFP) plasmid at 10 to 1 ratio was incubated with Lipofetamine®2000 and transfected to E18 primary rat cortical culture. The transfection efficiency was checked three days after transfection and the average efficiency was 5%. In the representative figure, red fluorescence demonstrated successful RFP expression while blue was DAPI, a nucleus stain. Image was obtained from epi-fluorescent microscope.

4.7.1 pUL37x1 Over-expression Reduced 6-OHDA Induced Apoptosis in Primary Rat Cortical Culture

The anti-apoptotic effect of pUL37x1 over-expression on primary rat cortical culture was similar to SH-SY5Y cells presented in section 4.3. Apoptosis was detected by the antibody against cleaved caspase-3, which is the activated form of caspase-3. Without 6-OHDA treatment, pUL37x1 over-expression did not increased

apoptosis compared with RFP over-expressing cells (RFP: 7.8±1.3, pUL37x1: 5.7±2.4, p>0.05). However, upon 20μM 6-OHDA treatment for 6 hours, pUL37x1 over-expression significantly reduced the percentage of apoptotic cells (RFP: 21.8±1.6, pUL37x1: 13.3±2.1, p<0.05) (Figure 4-26). These data provide the evidence that, like the SH-SY5Y cell model, pUL37x1 over-expression protected 6-OHDA induced apoptosis in the rat primary cortical culture model.

Figure 4-26

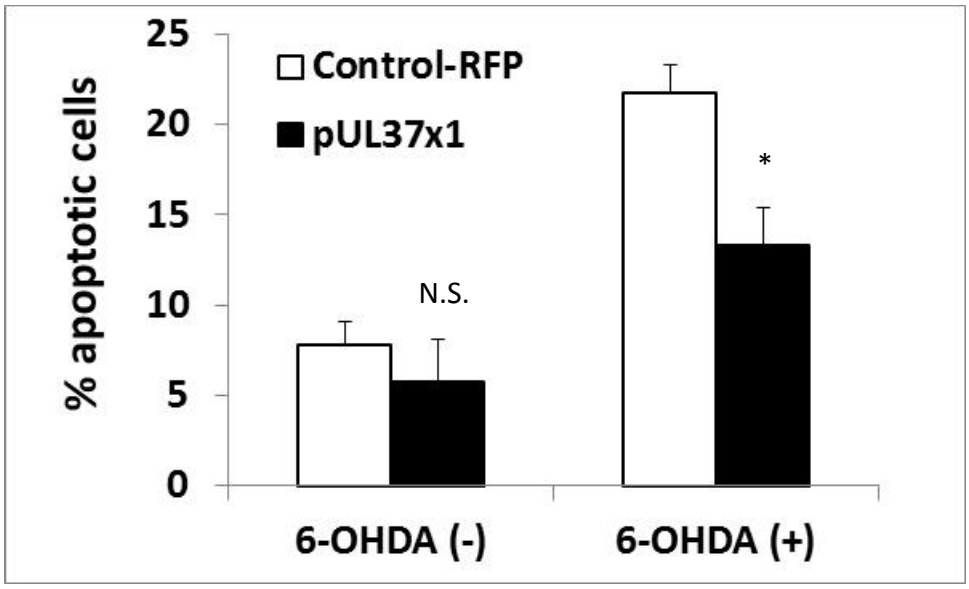

Figure 4-26. pUL37x1 expression in primary rat cortical culture cells significantly reduced the percentage of apoptotic cells induced by 20μM 6-OHDA treatment for 6 hours compared with RFP expressing ones (21.8±1.6% in RFP-control versus 13.3±2.1% in pUL37x1 over-expression, p<0.05, n=6). Data were presented as mean±S.E.M. Statistics was performed by two-tailed Student's t- test. (NS, non-significant, *,p<0.05)

4.7.2 pUL37x1 Over-expression Reduced Neuron-specific Death Induced by 6-OHDA in Primary Rat Cortical Culture

The main outcome of neuroprotection is evaluated by the ability of the intervention to decrease the neuronal death against toxin. Fluoro-jade C, a specific marker for degenerative neurons, is a good tool to investigate the protection. In primary rat cortical culture, pUL37x1 over-expression presented a similar trend of

protection to SH-SY5Y cells model. Without 6-OHDA treatment, over-expressing the viral protein did not increase neuronal death compared with RFP over-expression cells (RFP over-expression: 6.6±1.3, pUL37x1 over-expression: 9.9±0.5%, p<0.05). When challenged by 10μM 6-OHDA treatment for 24 hours, pUL37x1 over-expression significantly reduced the neuronal death labelled by FJ-C compared with RFP over-expression (RFP over-expression: 25.3±1.9, pUL37x1 over-expression: 17.4±2.7%, p<0.05) (Fig 4-27).

In conclusion, pUL37x1 over-expression reduced 6-OHDA induced apoptosis and neuronal death in rat primary cortical culture. These results strengthed the notion that modulation of mitochondria-dependent apoptosis could be neuroprotective in Parkinson's disease.

Figure 4-27

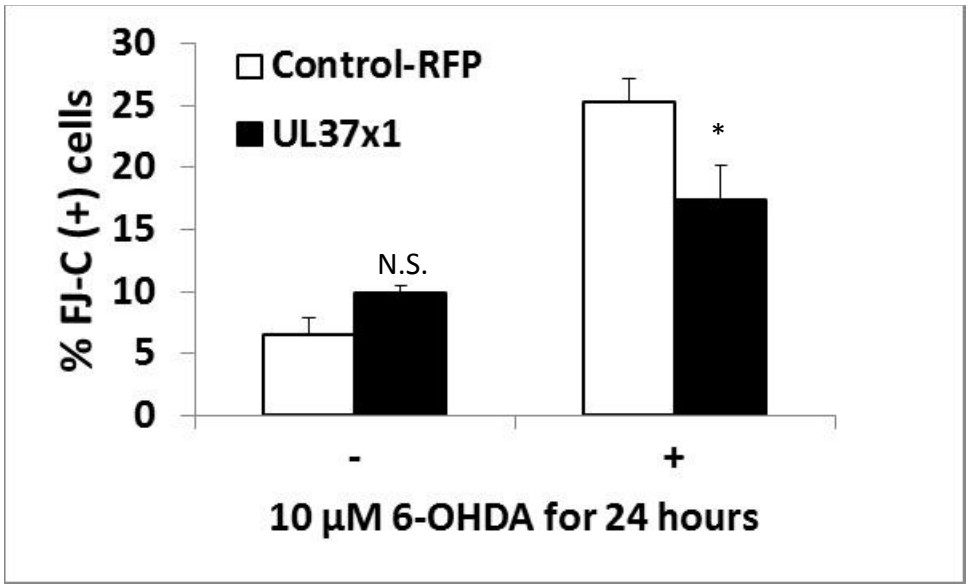

Figure 4-27 pUL37x1 expression in primary rat cortical culture cells significantly reduced the percentage of FJ-C positive neurons induced by 10μM 6-OHDA treatment for 24 hours compared with RFP expressing ones (25.3±1.9% in RFP-control versus 17.4±2.7% in pUL37x1 over-expression, p<0.05, n=6). Also, pUL37x1 over-expression did not significantly increase the cell death without 6-OHDA (RFP over-expression: 6.6±1.3, pUL37x1 over-

expression: 9.9±0.5%, n=4, p>0.05). Data were presented as mean±S.E.M. Statistics was performed by two-tailed Student's t- test. (NS, non-significant, *,p<0.05)

5 Discussion-Meclizine

5.1 Summary of Results of Meclizine

The present study demonstrated that meclizine, a glycolysis-enhancing agent, protected neuronal cells from 6-OHDA induced cytotoxicity. Meclizine exhibited cell death protection against 6-OHDA on both SH-SY5Y and primary rat cortical culture cells. The mechanism by which meclizine down-regulated 6-OHDA induced apoptosis involved mitochondrial hyperpolarization but not ROS scavenging. Meclizine prevented 6-OHDA induced mitochondrial depolarization, which is one of the key steps in apoptosis. This protective phenomenon was the same in both SH-SY5Y and primary rat cortical culture cells. Meclizine induced mitochondrial hyperpolarization resulted from glycolysis enhancement: co-administering glycolytic inhibitors with meclizine attenuated the hyperpolarization and cell death protection. However, it was unclear how meclizine increased glycolysis as it did not increase the protein level of several key glycolytic enzymes.

5.2 Meclizine Increased Glycolysis

Gohil *et al* were the first to report that meclizine skewed the balance of cellular glucose metabolism between OXPHOS and glycolysis and favoured the later. This effect was noted in several types of cell, including transformed cell lines and neuronal cells(Gohil et al., 2010). The present study utilized an XF analyser to detect the acidification of medium due to lactate synthesis, the end-product of glycolysis. It revealed that meclizine was able to increase the acidification in SH-SY5Y, a transformed neuroblastoma cell line. The increase of acidification lessened when co-applying glycolytic inhibitors. These results suggested that the acidification was

glycolysis-dependent and meclizine enhanced glycolysis as seen in the other types of cell. It is unlikely that measuring ECAR would truly reflect the glycolysis of neurons because of the mixed culture characteristics of primary rat cortical cultures. However, this report demonstrated that meclizine hyperpolarized mitochondria in primary rat cortical culture like SH-SY5Y cells, which indicated a glycolysis-enhancing ability of meclizine on primary neurons.

Traditionally, it is believed that neurons in the central nervous system dominantly rely on OXPHOS for ATP generation because 20% of oxygen consumption takes place in the brain which accounts for only 2% of body weight. Under conditions of mitochondrial inhibition, glycolysis is the main ATP source of neurons (Bauernfeind et al., 2013; Bolanos et al., 2010). It has also been postulated that, when OXPHOS is compromised, neurons are not able to up-regulate glycolysis sufficiently to meet the ATP demand, which results in a deficit of ATP, collapse of mitochondrial membrane potential and cell death (Almeida et al., 2001). However, Zala *et al* identified that the energy source for vesicle transport in axonal regions is independent from mitochondria and inhibition of mitochondrial ATP production does not significantly affect the transport. Moreover, inhibition or reduction of the key glycolytic enzyme, GAPDH blocks this transport (Zala et al., 2013). In addition, neurons are actually able to up-regulate glycolysis by either reduction of glycolytic enzyme degradation(Herrero-Mendez et al., 2009) or chemicals(Gohil et al., 2010). These findings indicate that glycolysis is essential for neuronal energy demand and neurons exhibit certain glycolytic reserves which can be up-regulated under stress conditions.

Glycolysis is often recognized as a less efficient method of ATP generation. Nevertheless, the rapid reactive rate of glycolysis compensates for the low-yield and enables the energy requirement to be met (Pfeiffer et al., 2001). In the present study, meclizine treatment alone did not increase apoptosis and cell death in primary rat cortical culture cells, which also supported the notion that shifting glucose metabolism from OXPHOS to glycolysis does not stress the neurons.

The mechanism by which meclizine increased glycolysis was unclear. HIF-1α which shunts pyruvates to lactate instead of allowing them to enter mitochondria was a major regulator of glucose metabolism. However, no increase of HIF-1α levels in cells treated with meclizine was found in a previous report(Gohil et al., 2010). The present study investigated the level of several key glycolytic enzymes following meclizine treatment, but failed to find any significant difference. There is a possibility that the increase of glycolysis was secondary to down-regulation of OXPHOS. Meclizine has been reported to inhibit phosphate cytidylyltransferase 2, ethanolamine (PCYT2) and lead to rapid accumulation of its substrate, phosphoethanolamine, which was itself an inhibitor of OXPHOS (Gohil et al., 2013).

5.3 Meclizine Hyperpolarized Mitochondria

Mitochondrial membrane potential is generated by the excretion of protons from the matrix to the intermembrane space. This electrical gradient is involved in several aspects of mitochondrial physiology, including ATP synthesis, calcium homeostasis and mitochondrial fusion/fission balance(Nicholls, 2004; Ishihara et al., 2003). Depolarization of mitochondria, a dysfunctional condition, is common in aging and Parkinson's disease(Nicholls, 2004; Abou-Sleiman et al., 2006). In addition,

dissipation of membrane potential is strongly associated with apoptosis. Loss of potential induces MMP and leads to initiation of the intrinsic pathway of apoptosis (Bernardi et al., 1999; Ly et al., 2003; Nicholls, 2004). Also, the maintenance of cytochrome c in the intermembrane space relies on this negative charged potential across the mitochondrial inner membrane. During apoptosis, depolarization of mitochondria and the subsequent remodelling of the mitochondrial matrix promotes the release of cytochrome c (Gottlieb et al., 2003; Uren et al., 2005).

That mitochondrial hyperpolarization prevents depolarization and apoptosis reveals a potential neuroprotective mechanism of meclizine. Physiologically, the potential difference is expended by the re-entrance of protons to convert ADP to ATP at complex V. Inhibition of complex V by oligomycin, without inhibiting the respiratory chain, blocks the proton re-entrance and strengthens the polarization across the mitochondrial intermembrane (Gottlieb et al., 2003; Otero and Carrasco, 1984). However, oligomycin reduces ATP synthesis from complex V and triggers apoptosis instead of protection(Li et al., 2004; Wolvetang et al., 1994; He et al., 2013). Another mechanism by which mitochondria can become hyperpolarized is to reverse the proton trafficking through complex V. When the intracellular ATP is sufficient, complex V consumes ATP to pump protons out rather than in(Iijima, 2006). The main source of extra-mitochondrial ATP generation is glycolysis. It is believed that cancer cells generate ATP predominantly from glycolysis even in oxygen abundant conditions, which is defined as the Warburg effect(Warburg, 1956). Owing to this alteration of glucose metabolism, cancer cells usually contain hyperpolarized mitochondria and are less sensitive to apoptosis(Bonnet et al., 2007;

Heerdt et al., 2005; Lugli et al., 2005). In contrast to complex V inhibition, mitochondrial hyperpolarization resulting from increased glycolysis would not lead to ATP deficit and cell death.

In the present study, meclizine has been proven to increase glycolysis in SH-SY5Y cells and, as a consequence, cause mitochondrial hyperpolarization. This effect was glycolysis-dependent rather than due to complex V inhibition: while co-administering glycolytic inhibitors with meclizine, both glycolysis enhancement and mitochondrial hyperpolarization were not seen. Meclizine also led to a similar effect in rat primary cortical culture cells: meclizine-treated primary cells exhibited hyperpolarization of the mitochondrial membrane potential compared with control.

This hyperpolarization phenomenon was the source of the cell death protection seen with meclizine treatment. CCCP, an uncoupler which affects the proton motive force, causes toxicity via the depolarization of mitochondria and initiation of apoptosis (Korlipara et al., 2004). The present study demonstrated that meclizine was not able to maintain mitochondrial membrane potential upon CCCP treatment and failed to protect against the cytotoxicity of CCCP compared with control. This indicated that the protection of meclizine was mitochondrial hyperpolarization-dependent.

5.4 Meclizine Reduced Apoptosis Induced by 6-OHDA

6-OHDA is a toxic oxidative metabolite of dopamine and the toxicity of 6-OHDA results from oxidative damage, including DNA damage, mitochondrial inhibition, and lipid peroxidation, which results in apoptotic cell death. In the SH-SY5Y model,

6-OHDA treatment causes reduction of glutathione, ATP depletion, mitochondrial cytochrome c release and activation of caspase(Gomez-Lazaro et al., 2008; Tirmenstein et al., 2005).

However, meclizine did not prevented 6-OHDA induced oxidative stress. In both non-treated and 6-OHDA treated cells, meclizine did not preserve aconitase activity, a parameter of oxidative damage due to its sensitivity to ROS. It has been postulated that aerobic glycolysis and down-regulation of OXPHOS reduces ROS generation(Brand and Hermfisse, 1997). However, Bolanõs *et al* argue that the increase of glycolysis flux leads to the down-regulation of the pentose phosphate pathway and, as a consequence, fewer endogenous anti-oxidants and reduced glutathione generation(Bolanos et al., 2010). As glycolysis-enhancement might exhibit both positive and negative impacts on cellular ROS level, it is not surprising that, in the present study, meclizine did not lessen the oxidative damage from 6-OHDA.

Mitochondrial depolarization is a universal and early phenomenon of 6-OHDA induced apoptosis (Tirmenstein et al., 2005; Lopes et al., 2010; Tovilovic et al., 2013) and numerous neuroprotective agents prevent this dissipation of mitochondrial membrane potential (Meng et al., 2013; Zhang et al., 2012; Huang et al., 2010). The aforementioned meclizine-induced mitochondrial hyperpolarization accounted for the protection against 6-OHDA induced apoptosis. Cells treated with meclizine retained normo-polarized mitochondria under 6-OHDA treatment, compared with 30% loss of mitochondrial membrane potential in control cells facing 6-OHDA challenging. Maintaining the pressure gradient upon apoptotic stimuli prevented

the increase of intracellular calcium, released due to the permeabilization of the mitochondrial membrane, and avoided the resulting mitochondrial cytochrome c release, which leads to amplification of the apoptotic signal and the 'point-of-no-return' in apoptotic cell death.

Activated caspase-3, the executor caspase, causes apoptosis and cell death. Meclizine did not directly inhibit the activation of caspase-3: in the absence of 6-OHDA, there was no change of caspase-3 activity in meclizine-treated cells compared with control. However, in 6-OHDA treated cells, meclizine modulated the apoptosis by mitochondrial hyperpolarization and blocked the process of apoptosis induced by 6-OHDA, which resulted in less caspase-3 activation in both SH-SY5Y and primary rat cortical cell cultures. The lowering the activation of caspase-3 by meclizine supported the hypothesis of its modulation of mitochondria-dependent apoptosis by glycolysis enhancement.

5.5 Meclizine Protected Cell Death from 6-OHDA

The ultimate goal of the present study was to test the protection by meclizine against cell death. Meclizine demonstrated neuroprotection against 6-OHDA induced cell death on SH-SY5Y and primary rat cortical culture cells and this reduction was glycolysis-dependent: glycolytic inhibitors, which resulted in less lactate synthesis, attenuated the protection.

It is worth mentioning the significance of loss of neurons, especially nigral dopaminergic neurons, in Parkinson's disease. Degeneration of more than half of the nigral dopaminergic neurons is thought to lead to the manifestation of the early

motor symptoms of Parkinson's disease(Marsden, 1990). L-dopa treatment is effective at the beginning of disease, because the remaining dopaminergic neurons are able to convert the L-dopa to dopamine and buffer the sudden change of dopamine levels in the synapse. However, the progression of neuronal loss results in a reduction in this buffering, which causes the drug-related motor complications associated with long-term treatment(Obeso et al., 2000). 6-OHDA, a widely-used toxin in Parkinson's disease models, also induces neuronal death *in vivo* and *in vitro*(Drechsel and Patel, 2008). The present study successfully demonstrated a protective effect of meclizine against 6-OHDA induced cell death. This protection was not only shown on SH-SY5Y, a transformed neuroblastoma cell line, but also on primary rat cortical culture cells. Primary cells, unlike transformed cell line, are generally more vulnerable to toxin-induced cell death and more difficult to protect. The protective effect of meclizine obtained from primary rat cortical culture supported the hypothesis that modulation of mitochondria-dependent apoptosis may be neuroprotective in Parkinson's disease. In addition, by using FJ-C, a specific fluorescent neuronal degeneration marker, it was possible to identify the dead neurons only and overcome the issues associated with using a mixed culture like rat primary cortical cultures. The observation that fewer neurons were stained by FJ-C when they were challenged by 6-OHDA in the presence of meclizine is evidence of neuroprotection.

It is still important to measure cell death protection after confirmation of anti-apoptotic effect. In theory, cell death follows two major pathways: apoptosis and necrosis. In contrast to apoptosis, necrosis presents with different features, such as

cellular swelling leading to bursting of the plasma membrane and release of the cytosolic contents to the surrounding tissue, which causes an inflammatory response (Golstein and Kroemer, 2007). There is a balance between these two distinct mechanisms of cell death and on some occasions, down-regulation of apoptosis can promote necrosis. Kalai and colleagues have demonstrated that caspase inhibition or over-expression of Bcl-2 prevents apoptosis but promotes necrosis(Kalai et al., 2002). Meilhac *et al* also showed that over-expression Bcl-2 triggers necrosis and does not protect against cell death induced by oxidized low density lipoproteins(Meilhac et al., 1999). Fortunately, meclizine down-regulated apoptosis but is unlikely to affect the levels of necrosis: the anti-apoptosis effect was in line with the reduction of cell death induced by 6-OHDA.

5.6 Implications of Neuroprotection by Meclizine

The present study demonstrated the neuroprotective effect of meclizine in Parkinson's disease cell models. It revealed that increased glycolysis protected the neurons from 6-OHDA induced cytotoxicity. Although some articles have previously described the neuroprotective benefit of glycolysis (Newington et al., 2011; Newington et al., 2012; Williams et al., 2007), this alternative ATP synthesis pathway receives little attention in the field of neuroprotection. However, it has long been suggested that cancer cells, which are very resistant to apoptosis and cell death, exhibit very high glycolytic activity. The alteration of glucose metabolism is thought to be responsible for this resistance and, therefore, glycolytic inhibition results in the sensitization of chemotherapy and apoptosis(Loar et al., 2010; Vuyyuri et al., 2013). For this reason increased glycolysis should not be ignored as a

neuroprotective approach for Parkinson's disease, an apoptosis-related degenerative disease.

Another implication of the present study is the protective role of mitochondrial hyperpolarization. Despite some evidence suggesting that mitochondrial depolarization is not a mandatory process during apoptosis (Ly et al., 2003; Scarlett et al., 2000), hyperpolarization of mitochondria by meclizine was proved to be an anti-apoptotic approach in Parkinson's disease models. Physiologically, astrocytes and myocytes are able to up-regulate glycolysis in order to maintain mitochondrial membrane potential and resist apoptosis, whereas neurons are more sensitive to respiratory inhibition or mitochondrial dysfunction as they lack this glycolysis-enhancement ability (Almeida et al., 2001; Yao et al., 2011). However, meclizine enhanced glycolysis in neurons and helped to maintain mitochondrial membrane potential upon exposure to 6-OHDA, which was a protective strategy for the pathogenesis of Parkinson's disease, a mitochondrial dysfunction-associated disease.

The major advantage of meclizine is that it has been prescribed to patients for decades and has a good safety and tolerability profile. Meclizine can be applied to either genetic or toxin-induced Parkinson's disease animal models directly after confirmation of neuroprotection *in vitro*. In fact, meclizine has been shown to reduce the volume of infarction in rat stroke model, and to prevent neuronal dystrophy and cell death in *Caenorhabditis elegans* and *Drosophila melanogaster* models of polyglutamine toxicity(Gohil et al., 2010; Gohil et al., 2011). It is

therefore hoped that meclizine will reduce the nigral dopaminergic neuronal loss and lessen the parkinsonism symptoms in experimental animals.

5.7 Limitations and Future Work on Meclizine

Despite the promising results, the present study exhibited some limitations about the use of meclizine. High doses of meclizine increased the spontaneous cell death in both SH-SY5Y and primary rat cortical culture cells. This toxicity might have resulted from the over-activation of glycolysis which compromised the pentose phosphate pathway and reduced the synthesis of glutathione, an endogenous anti-oxidant. Similar conditions have been mentioned by Herrero-Mendez *et al*, who claimed that moderate 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase isoform 3 over-expression is protective whereas higher expression levels leads to rapid death of the neurons by apoptosis(Herrero-Mendez et al., 2009). In future studies testing meclizine for neuroprotection in animal studies or clinical trials, an optimal dose which avoids this toxicity needs to be identified first.

The other problem identified is clarification of the mechanism by which meclizine increases glycolysis. Although the protein level of glycolytic enzymes did not alter in the present study, some post-translational modifications of the enzymes, which could affect the glycolytic activity without changing the gross protein level, could occur and thus required further investigation(Smerc et al., 2011; Mathupala et al., 1997). Furthermore, as well as HIF-1α, there are some other key regulators that modulate glucose metabolism. For instance, activation of receptor tyrosine kinase/PI3K/AKT/mammalian target of rapamycin signalling cascade could induce aerobic glycolysis through up-regulation of pyruvate kinase M2 and other glycolytic

enzymes(Sun et al., 2011). Therefore, it is worth investigating these glycolytic profiles to elucidate the full mechanism of glucose metabolism alteration by mecilizine.

It is essential to mention about the drawbacks of using toxin-induced model in the research of Parkinson's disease. A major problem with the classic toxin-induced models is that they reproduce parkinsonism but not always Parkinson's disease, a neurodegenerative disease with the presence of Lewy body inclusion in peripheral and central nervous system. 6-OHDA and MPTP fail to induce the synuclein aggregation and Lewy body whereas intravenous rotenone infusion in rat led to the cytoplasmic inclusion(Beal, 2010). Menwhile, the acute insult from toxin, for example, 6-OHDA, unliken the slowly progressive characteristic of Parkinson's disease. For 6-OHDA, the other problem is the administration. Striatal injection of 6-OHDA induces degeneration of nigral dopaminergic neurons but fail to produce a widespread neuronal degeneration which had been noted in Parkinson's disease(Braak et al., 2003a).

6 Discussion-pUL37x1

6.1 Summary of Results of pUL37x1

The present study demonstrated the neuroprotective effect of pUL37x1 over-expression against staurosporine and 6-OHDA-induced apoptosis and cell death in both SH-SY5Y and primary rat cortical cultured cells. pUL37x1 provided two different neuroprotective strategies simultaneously, both of which modulated mitochondria-dependent apoptosis. The first one was Bax-dependent. pUL37x1, located on mitochondrial outer membrane, led to mitochondrial translocalization and inactivation of Bax. Silencing of Bax in control cells reduced the staurosporine-induced cell death but failed to provide further protection on pUL37x1 over-expressing cells. The other strategy was to hyperpolarize mitochondria by increased glycolysis. pUL37x1 over-expression increased glycolysis which led to hyperpolarized mitochondria and blocked 6-OHDA induced depolarization and further apoptotic processes. Mitochondrial hyperpolarization and cell death protection was attenuated or lost if glycolytic inhibitors were co-administered. These results support the concept that controlling mitochondria-dependent apoptosis is neuroprotective in Parkinson's disease.

6.2 The pUL37x1 Over-expressing Lines

pUL37x1 is a protein from human CMV. To the best of my knowledge, there is no endogenous pUL37x1 found in humans. In order to investigate the protection of over-expressing pUL37x1 in SH-SY5Y cells, stable pUL37x1 expressing lines were required. Stable expressing lines were defined as the cell's genome with foreign gene integration and daughter cells of the transfected cell also expressed the new

gene. The advantages of using stable expression instead of transient transfection include long-term gene expression, consistency and the option of further genetic manipulation(Sparkes et al., 2006). After antibiotic selection and pUL37x1-HA expression confirmation by western blot analysis, several colonies were identified as stable over-expressing lines. However, because of the nature of random genome integration, some colonies could alter basal cellular protein expression. Choosing three lines instead of one eliminates the impact of possible cloning artefacts.

As there was no reliable anti-pUL37x1 antibody available commercially, an HA-tag was conjugated to the C-terminus of pUL37x1 in the transfection construct for detection purposes. In the present study, anti-HA antibody detected pUL37x1-HA bands from the stable over-expressing lines at 60kD by western blot analysis. Densitometry analysis revealed that the three lines expressed different levels of pUL37x1, and the difference between maximum with minimum was nearly one fold change. However, in the following experiments, there was no significant difference between three lines in cell death protection, anti-apoptosis and other effects. This lack of difference was probably because even the lowest expression level of pUL37x1 was above the effective dosage.

The confirmation of over-expression purity relied on an immunocytochemistry assay even though the pUL37x1 over-expressing lines had gone through antibiotic selection and protein expression, had been confirmed. It is essential to establish this purity because following experiments measured gross cellular outcomes, such as cell death, apoptosis and expression level of significant proteins. These results would be unreliable if the cells exhibited a pronounced variation in their over-

expression. However, all three lines presented a homogenous pUL37X1-HA expression. Immunocytochemistry results also confirmed the mitochondrial localization of pUL37x1 in SH-SY5Y cells. Goldmacher *et al* noted the mitochondrial localization of pUL37x1 in transfected HeLa cells(Goldmacher et al., 1999) and the first domain of pUL37x1 has been identified as responsible for mitochondria targeting and localization(Hayajneh et al., 2001). This similarity indicates that in present study, pUL37x1 in SH-SY5Y cells behaved in the same manner as previous results obtained from other cell types.

As well as normal SH-SY5Y cells, two other stable over-expressing cell lines were also used as controls. The reason for including another two stable over-expressing controls was to mimic the condition in pUL37x1 over-expressing lines. The first of these control cell lines stably over-expressed dsRed-Mito, a mitochondrial-localized fluorescent protein without altering mitochondrial function. It resembles the mitochondria-localized characteristic of pUL37x1. The other one was a stable pcDNA empty vector over-expressing line, which was the same plasmid that carried pUL37x1 genes during transfection.

6.3 pUL37x1 Over-expression Reduced Drug Induced Apoptosis and Cell Death

The anti-apoptotic property of pUL37x1 has been mentioned in the introduction (section 1.7.2). In this study, two apoptosis inducers were used to test the protective effect of pUL37x1 over-expression. 6-OHDA has been described in section 5.4. The other toxin was staurosporine, a protein kinase C inhibitor which is isolated from the culture broth of *Streptomyces staurospores*. Staurosporine

activates Bax and triggers both caspase-dependent and independent apoptotic pathways (Zhang et al., 2004; Belmokhtar et al., 2001). Staurosporine-challenge tests the anti-apoptotic effect of pUL37x1 in neuronal cells, whereas 6-OHDA provides a neurodegenerative model to investigate the neuroprotection.

The release of cytochrome c from mitochondria to cytoplasm results from MMP. Cytoplasmic cytochrome c interacts with Apaf-1 protein to form an apoptosome and activates caspase. Hence, both the release of cytochrome c and the activation of caspase, especially caspase-3, are the hallmarks of apoptosis processing. Down-regulating MMP reduces mitochondrial cytochrome c release upon apoptotic stimuli and down-regulates the following caspase activation related cell death (Yang et al., 1997; Koya et al., 2000). pUL37x1 over-expressing SH-SY5Y cells reduced the release of cytochrome c and activation of caspase-3 upon staurosporine treatment compared with control. Also, pUL37x1 down-regulated casapse-3 activation in both SH-SY5Y and primary rat cortical cultured cells. These results support the hypothesis that pUL37x1 is anti-apoptotic in neuronal cells and neuroprotective in Parkinson's disease cellular models.

The next crucial question is whether pUL37x1 over-expression provides protection of neuronal death. The significance of neuronal death in Parkinson's disease and the possible discrepancy between apoptosis and cell death has been mentioned in section 5.5. Not surprisingly, pUL37x1 provided an anti-apoptotic effect to prevent cell death against staurosporine and 6-OHDA. In SH-SY5Y cells, the protection presented under several different conditions, including high dose, short-term toxin treatment and moderate dose, long-term toxin treatment. In rat primary cortical

culture cells, pUL37x1 transfection protected neurons from 6-OHDA induced death. This evidence of anti-apoptosis and cell death protection from pUL37x1 over-expression supports the original hypothesis.

6.4 pUL37x1 Over-expression Modulated Bax-related Apoptosis

The involvement of Bax in apoptosis has been describe in section 1.4.1.2. In brief, activated BH3- only proteins induce Bax oligomerazation and mitochondria-translocalization of Bax. The Bax oligomers insert into the mitochondrial outer membrane and lead to MMP. Bax also plays a role in apoptosis in Parkinson's disease. Tatton *el al* found that a greater number of Bax-immunopositive cells are observed in Parkinson's disease nigral sections compared to aged, control nigral sections(Tatton, 2000). Hartmann and colleagues also demonstrated that in Parkinson's disease brains the percentage of Bax-positive melanized nigral neurons containing Lewy bodies is significantly higher than the overall percentage of Bax-positive neurons among melanized neurons(Hartmann et al., 2001a). Also, parkin modulates the ubiquitination of Bax and prevents basal and apoptotic stress-induced translocation of Bax to the mitochondria(Johnson et al., 2012).

Inactivation of Bax has been found to down-regulate apoptosis. Nicotine enhances the Ser^{184} phosphorylation of Bax by Akt and results in the failure of activation and mitochondria-translocalization of Bax (Gardai et al., 2004; Xin and Deng, 2005). pUL37x1 is known to cause the mitochondria-translocalization of Bax, which also takes place during apoptosis. However, this translocalization leads to a conformational change and inactivation of Bax, as mentioned in section 1.7.2. In the present study, pUL37x1 over-expression led to mitochondria-translocalization

of Bax in the absence of toxin. This translocalization was shown to not result in apoptosis because there was no increase of cytochrome c release or caspase-3 activation under these conditions. This indicated that in stable pUL37x1 over-expressing SH-SY5Y cells, the ectopic expressing protein induced Bax translocalization to mitochondria without the initiation of apoptosis, which is in accordance with previous studies.

Bax siRNA silencing provides a way to investigate the Bax-dependent protection of pUL37x1 over-expression. Bax ablation has been demonstrated to have an anti-apoptotic and neuroprotective effect. Vila *et al* show that Bax deficient mutant mice were resistant to MPTP neurotoxicity (Vila et al., 2001). In the present study, Bax siRNA silencing for 3 days caused 80% reduction of Bax protein levels, compared with Scr siRNA, in both control and pUL37x1 over-expressing cells. The control group benefitted from cell death protection against staurosporine with Bax siRNA silencing, whereas pUL37x1 over-expressing cells did not. This suggested that the strength of Bax inactivation by pUL37x1 over-expression was equal to the ablation of four-fifths of the intracellular Bax. In addition, the protection of pUL37x1 was shown to be Bax-dependent. Combining these two pieces of data, it can be concluded that, in neuronal cells, pUL37x1 over-expression translocalizes and inactivates Bax on the mitochondrial outer membrane, and this is the mechanism responsible for the anti-apoptotic effect.

6.5 pUL37x1 Over-expression Modulated Apoptosis by Glycolysis-Induced Mitochondrial Hyperpolarization

CMV infection alters cellular metabolism in host cells. Munger *et al* found that the levels of metabolites involved in glycolysis, the citric acid cycle, and pyrimidine nucleotide biosynthesis markedly increased after CMV infection measuring by liquid chromatography-tandem mass spectrometry (Munger et al., 2006). Later, the same group also demonstrated the increase of glycolytic flux during CMV infection (Munger et al., 2008). These findings indicated that CMV infection is associated with up-regulation of glycolysis. The present study showed that pUL37x1 over-expression markedly up-regulated glycolysis. This increase did not result from inhibition of OXPHOS: there was no impairment of either oxygen consumption rate or ADP phosphorylation activity from pUL37x1 over-expressing cells. In fact, the glycolysis-enhancement effect led to the hyperpolarization of the mitochondrial membrane potential and prevented 6-OHDA induced mitochondrial depolarization. Glycolytic inhibitors attenuated the hyperpolarization and cell death prevention provided by pUL37x1 over-expression, which indicated that part of the protection of pUL37x1 was glycolysis-dependent. It is a novel finding that pUL37x1 controlled mitochondria-dependent apoptosis not only relies on the well-known Bax inactivation, but also mitochondrial hyperpolarization. That pUL37x1 can present two different approaches to MMP modulation during apoptotic stimuli could be particularly relevant to neuroprotection.

However, the present study failed to identify the mechanism by which pUL37x1 increased glycolysis. There was no alteration in the protein levels of several

essential glycolytic enzymes. There remains the possibility that pUL37x1 over-expression does not directly up-regulate glycolysis, but acts through another signal transduction pathway. For instance, CMV infection increases glycolysis by activation of calmodulin-dependent kinase kinase (CaMKK) and, inhibition of CaMKK severely attenuates production of viral progeny and blocked viral DNA replication(McArdle et al., 2011). CaMKK up-regulates glycolysis through activation of either CaM kinase I or AMP-activated kinase to phosphorylate phosphfructokinase-2, a key rate-determine enzyme of glycolysis (reviewed by (Rider et al., 2004; Towler and Hardie, 2007). Increased intracellular calcium is able to activate CaMKK and it has been shown that pUL37x1 can cause the release of Ca^{2+} from the endoplasmic reticulum into the cytosol (Sharon-Friling et al., 2006). This association leads to the speculation that pUL37x1 over-expression could increase glycolysis through a calcium and CaMKK-dependent pathway, a hypothesis supported by the results from the present study where pUL37x1 over-expression increased glycolysis without changing the protein level of glycolytic enzymes.

6.6 Implications on Neuroprotection of pUL37x1

The encouraging results of neuroprotection by pUL37x1 over-expression suggests that modulating mitochondria-dependent apoptosis is a promising approach to prevent nigral dopaminergic neuronal loss in Parkinson's disease. Moreover, pUL37x1 provides more than one anti-apoptotic mechanisms to confer neuroprotection. As described in the section 1.5.1, MMP can be manipulated by several factors. pUL37x1 is superior to other agents in the modulation apoptosis because of its dual anti-apoptotic effect: Bax inactivation and mitochondrial

hyperpolarization by glycolysis. It has similarities to the concept of 'cocktail' therapy, which has been widely used in the treatment of cancer, autoimmune diseases and human immunodeficiency virus infection. The idea of cocktail therapy in neuroprotection of Parkinson's disease has been raised due to the multi-factorial characteristic of the disease pathogenesis (Hirsch, 2007). With two protective mechanisms, one can compensate for the other's drawbacks. For instance, pUL37x1 is able to inhibit Bax-related apoptosis but not Bak, the other pro-apoptotic Bcl-2 family protein (Arnoult et al., 2004). Mitochondrial hyperpolarization boosted by glycolysis covers this deficit due to its more general effect on modulating mitochondria-dependent apoptosis. In addition, this strategy would be able to reduce the toxicity and side effects from a high dose-single approach.

Furthermore, the present study is one of the first to test the neuroprotective effects of viral anti-apoptosis products. Numerous anti-apoptotic strategies have been noted from viruses that allow them to escape apoptosis by the host cell (reviewed by (Benedict et al., 2002), and utilizing a safe viral protein to prevent apoptosis-related neurodegeneration provided may provide opportunities for researchers to achieve the goal of neuroprotection in the future.

6.7 Limitations and Future work of pUL37x1

Despite the promising results of neuroprotection by pUL37x1, there are still some limitations with the present study. The first issue is the use of 6-OHDA to induce neuronal apoptosis and death as the use of toxin-induced models in Parkinson's disease is still of uncertain relevance . 6-OHDA increases oxidative stress and local injection of 6-OHDA in substantia nigra causes loss of dopaminergic neurons.

However, it fails to produce the core pathological feature of Parkinson's disease, Lewy body formation, in experimental animals and this raises the question as to whether the 6-OHDA model is able to reflect the real pathogenesis of Parkinson's disease. Genetic Parkinson's disease models provide an alternative. However, they also trigger debate because genetic mutations account for less than 10% of the etiology of Parkinson's disease. Moreover, some mutation models do not lead to Lewy body formation or progressive loss of nigrostriatal dopaminergic neurons(review by (Chesselet et al., 2008).

Another issue with the present study is the use of primary rat cortical culture instead of midbrain culture while testing the protective effect. There is no doubt about the significance of nigral dopaminergic neuron loss in Parkinson's disease. However, the low-yield of tyrosine hydroxylase positive neurons in the culture and highly variable nature of midbrain culture limits the application. In addition, plenty of evidence, as mentioned in previous sections, shows that there is widespread neuronal degeneration in the whole brain as the disease progress, which supports the use of cortical cultures to test the protective effect of pUL37x1 over-expression. In the future, the protection of pUL37x1 could be tested on induced pluripotent stem cell-differentiated ventral midbrain dopaminergic neurons, which provides a more powerful Parkinson's disease cellular model.

The next stage of testing the neuroprotection provided by pUL37x1 expression is in animal studies. However, this generates another concern about the use of pUL37x1. Viral vectors are required to express pUL37x1 in neurons and the safety of viral vectors and pUL37x1 would be an issue. Fortunately, there has been no

troubling safety problem reported in recent decade of gene therapy trials of Parkinson's disease (review by (Bartus et al., 2014). Added to this, CMV is recognized as a low-virulence virus and only causes severe infection in immune-compromised patients. Therefore, in terms of toxicity, pUL37x1 treatment is expected to be minimal.

In conclusion, pUL37x1, a protein with dual anti-apoptotic mechanisms, is a promising agent to protect against the neuronal degeneration associated with Parkinson's disease. It is worth investigating the neuroprotection of pUL37x1 in experimental animals in the future.

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Supplementary Data

S1. pUL37x1 over-expression did not increase spontaneous cell death but protected 6-OHDA and staurosporine-induced cytotoxicity: presented by individual control and pUL37x1 over-expressing line

S1-1

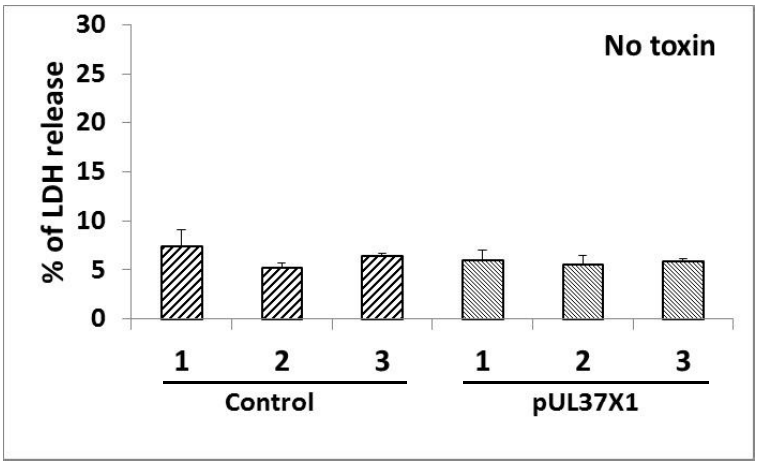


Figure S1-1. pUL37x1 over-expressing SH-SY5Y lines did not increase spontaneous cell death in no-toxin status compared with control. Data were presented as mean±S.E.M. Statistics was performed by one-way ANOVA with Dunnett's post-hoc analysis. (n=6)

S1-2

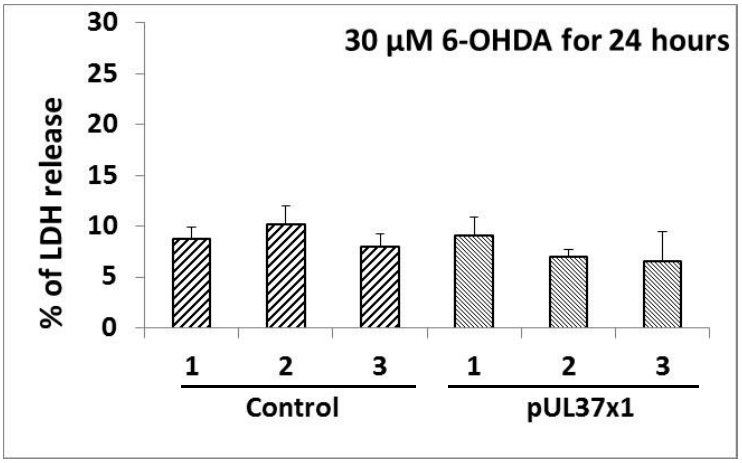
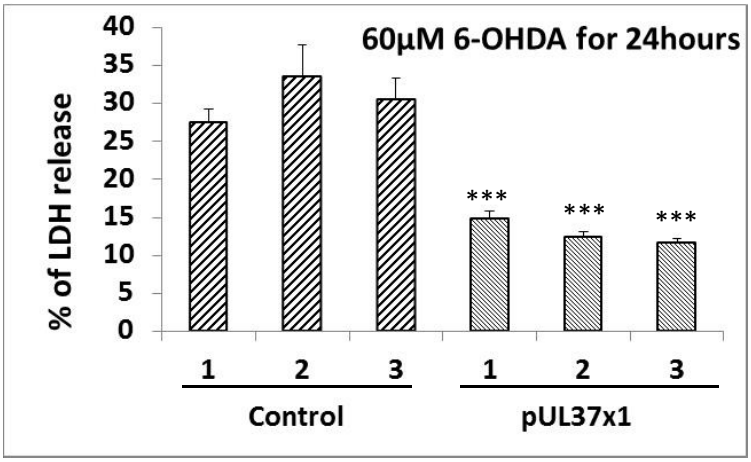


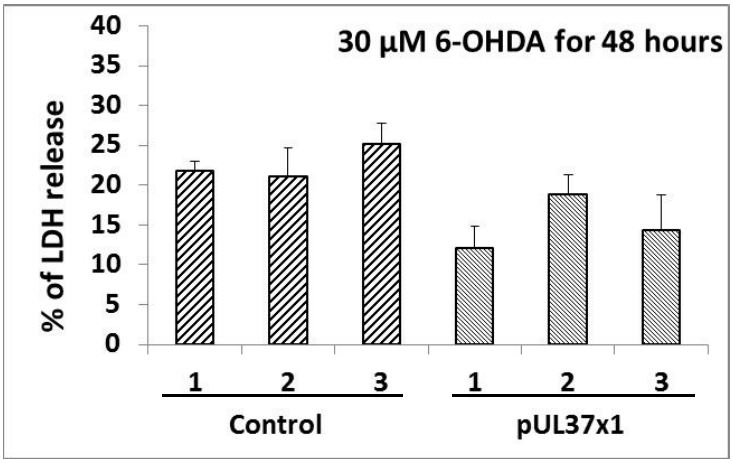
Figure S1-2. 30µM 6-OHDA treatment for 24 hours caused less than 10% of LDH release in SH-SY5Y cells (control-1). There was no significant difference between control and pUL37x1 over-expressing cells in the percentage of LDH release. Data were presented as mean±S.E.M. Statistics was performed by one-way ANOVA with Dunnett's post-hoc analysis. (n=6

S1-3



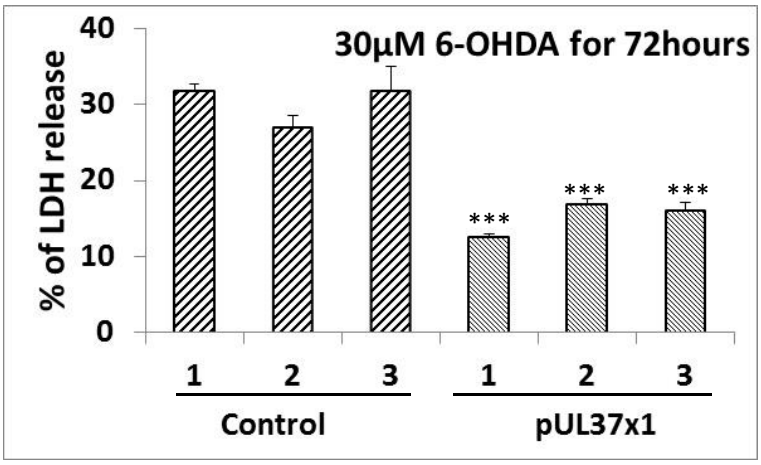
S1-3. pUL37x1 over-expression reduced the percentage of LDH release compared with control SH-SY5Y cells (control-1). All three lines exhibited significant protection against 6-OHDA. Data were presented as mean±S.E.M. Statistics was performed by one-way ANOVA with Dunnett's post-hoc analysis. (n=6, ***, p<0.001)

S1-4



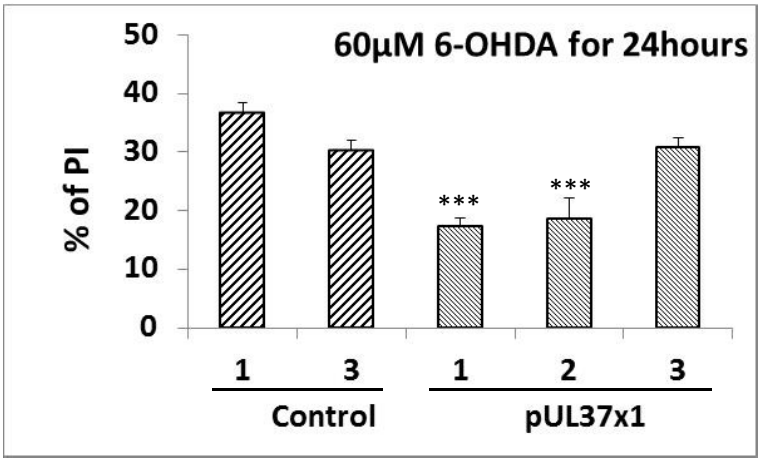
S1-4. pUL37x1 over-expression reduced the percentage of LDH release against 30µM 6-OHDA treatment for 48 hours compared with SH-SY5Y cells (control-1). Data were presented as mean±S.E.M. Statistics was performed by one-way ANOVA with Dunnett's post-hoc analysis. (n=8)

S1-5



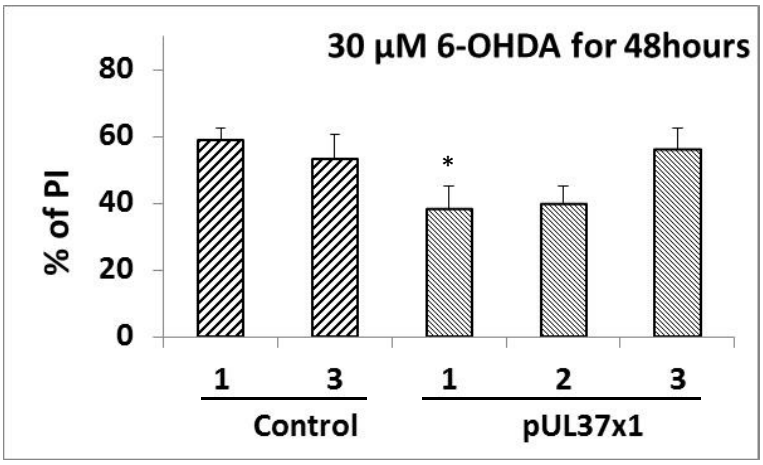
S1-5. pUL37x1 over-expression reduced the percentage of LDH release against 30μM 6-OHDA treatment for 72 hours compared with control SH-SY5Y cells (control-1). All three lines exhibited significant protection against 6-OHDA. Data were presented as mean±S.E.M. Statistics was performed by one-way ANOVA with Dunnett's post-hoc analysis. (n=6, ***, p<0.001)

S1-6



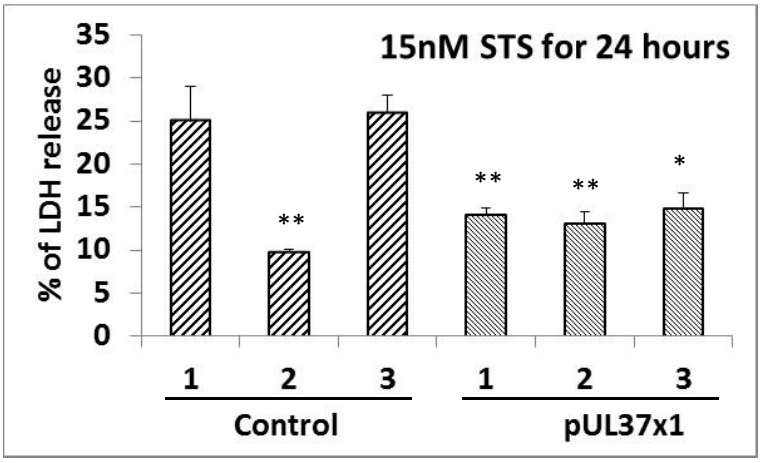
S1-6. pUL37x1 over-expression reduced the percentage of PI fluorescence against 60μM 6-OHDA treatment for 24 hours compared with control SH-SY5Y cells (control-1). pUL37x1-1 and 2 lines exhibited significant protection against 6-OHDA. Data were presented as mean±S.E.M. Statistics was performed by one-way ANOVA with Dunnett's post-hoc analysis. (n=6, ***, p<0.001)

S1-7



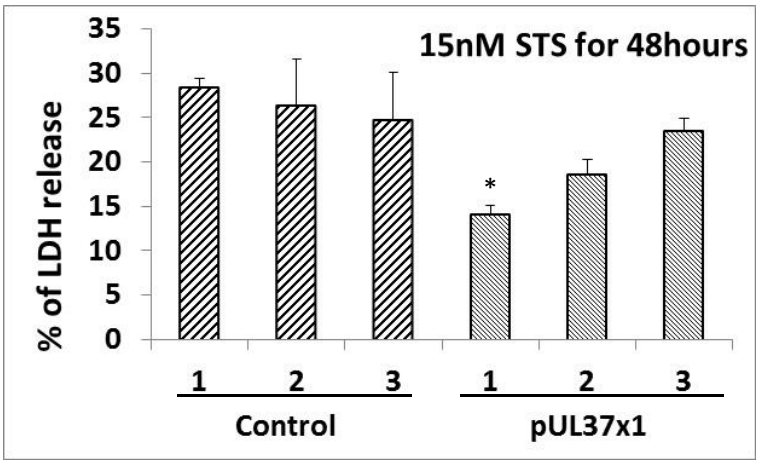
S1-7. pUL37x1 over-expression reduced the percentage of PI fluorescence against 30µM 6-OHDA treatment for 48 hours compared with control SH-SY5Y cells (control-1). pUL37x1-1 line exhibited significant protection against 6-OHDA. Data were presented as mean±S.E.M. Statistics was performed by one-way ANOVA with Dunnett's post-hoc analysis. (n=6, *, p<0.05)

S1-8



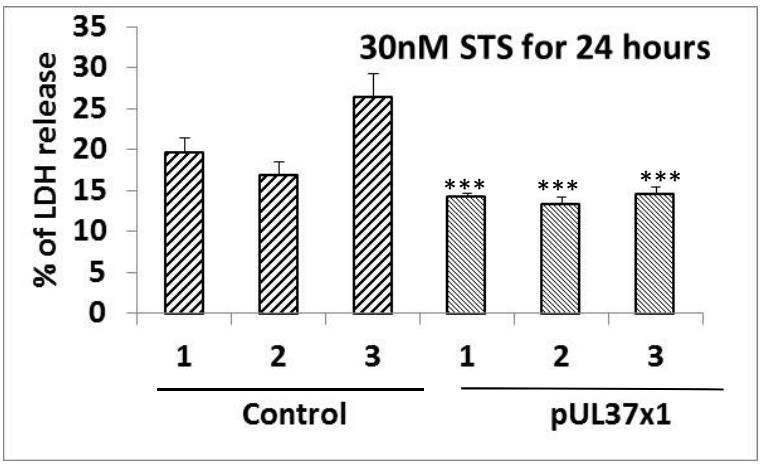
S1-8. pUL37x1 over-expression reduced the percentage of LDH release against 15nM staurosporine treatment for 24 hours compared with control SH-SY5Y cells (control-1). All three pUL37x1 over-expressing line exhibited significant protection against staurosporine. Data were presented as mean±S.E.M. Statistics was performed by one-way ANOVA with Dunnett's post-hoc analysis. (n=4, *, p<0.05, **, p<0.01)

S1-9



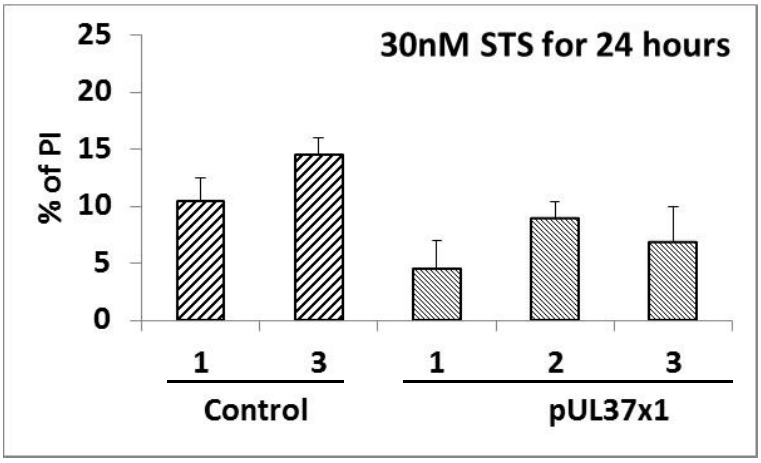
S1-9. pUL37x1 over-expression reduced the percentage of LDH release against 15nM staurosporine treatment for 48 hours compared with control SH-SY5Y cells (control-1). pUL37x1-1 over-expressing line exhibited significant protection against staurosporine. Data were presented as mean±S.E.M. Statistics was performed by one-way ANOVA with Dunnett's post-hoc analysis. (n=6, *, p<0.05)

S1-10



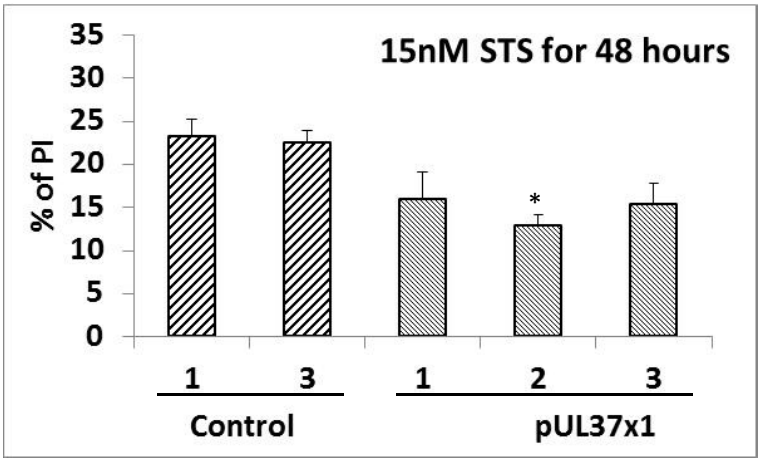
S1-10 pUL37x1 over-expression reduced the percentage of LDH release against 30nM staurosporine treatment for 24 hours compared with control SH-SY5Y cells (control-1). All pUL37x1 over-expressing lines exhibited significant protection against staurosporine. Data were presented as mean±S.E.M. Statistics was performed by one-way ANOVA with Dunnett's post-hoc analysis. (n=8, ***, p<0.001)

S1-11



S1-11. pUL37x1 over-expression reduced the percentage of PI fluorescence against 30nM staurosporine treatment for 24 hours compared with control SH-SY5Y cells (control-1). Data were presented as mean±S.E.M. Statistics was performed by one-way ANOVA with Dunnett's post-hoc analysis. (n=6)

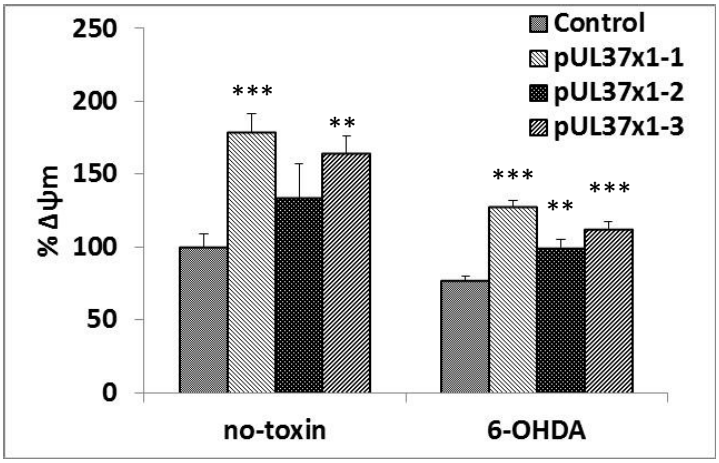
S1-12



S1-12. pUL37x1 over-expression reduced the percentage of PI fluorescence against 15nM staurosporine treatment for 48 hours compared with control SH-SY5Y cells (control-1). pUL37x1-2 over-expressing line exhibited significant protection against staurosporine. Data were presented as mean±S.E.M. Statistics was performed by one-way ANOVA with Dunnett's post-hoc analysis. (*, p<0.05, n=6)

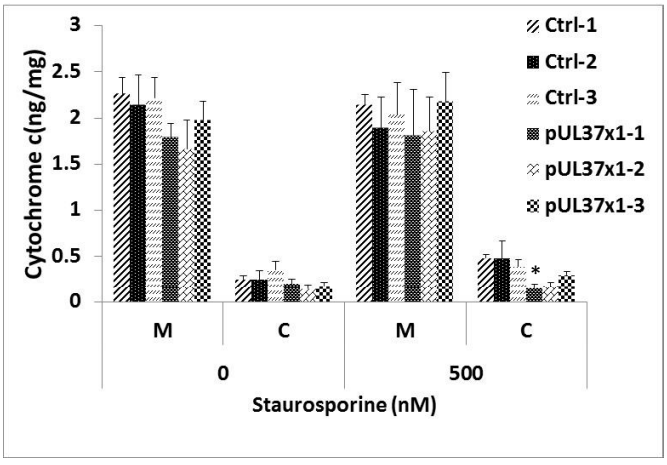
S2 pUL37x1 over-expression modulated mitochondria-dependent apoptosis-presented by individual control and pUL37x1 over-expressing line

S2-1



S2-1. pUL37x1 over-expression hyperpolarized mitochondria compared with control SH-SY5Y cells. pUL37x1-1 and 3 lines significantly hyperpolarized mitochondrial membrane potential in no-toxin status whereas all three over-expressing lines significantly maintained mitochondrial membrane potential upon 100µM 6-OHDA treatment for 1 hour. Mitochondrial membrane potential was measured by TMRM fluorescence and normalized by the average of TMRM fluorescence obtained from no-toxin SH-SY5Y cells. Data were presented as mean±S.E.M. Statistics was performed by one-way ANOVA with Dunnett's post-hoc analysis. (**, p<0.01, ***, p<0.001)

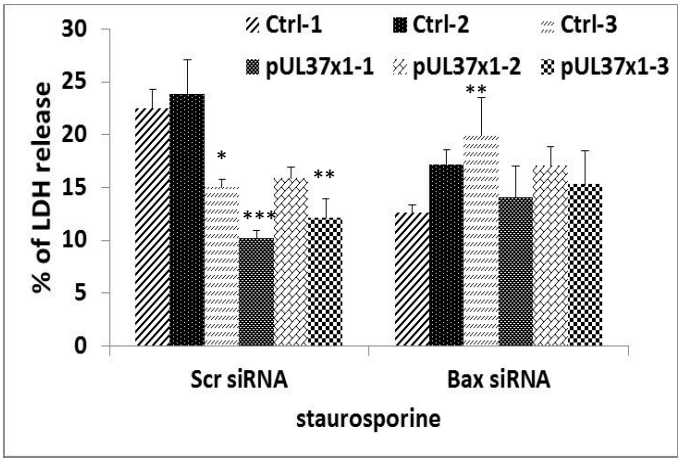
S2-2



S2-2. pUL37x1 reduced the cytochrome c release upon 500nM staurosporine treatment for 3 hours. pUL37x1-1 line demonstrated a significant reduction of cytosolic cytochrome c concentration upon 500nM staurosporine treatment for

3 hours compared with control SH-SY5Y cells. Data were presented as mean±S.E.M. Statistics was performed by one-way ANOVA with Dunnett's post-hoc analysis. (*, $p<0.05$)

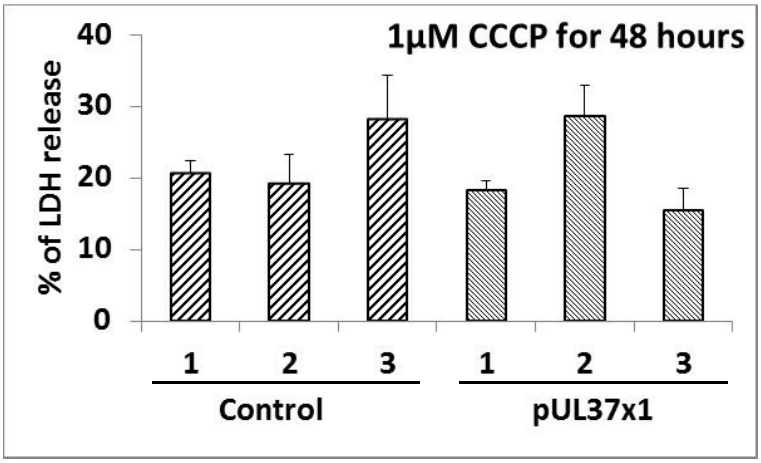
S3. The protection of pUL37x1 over-expression was Bax-dependent: presented by individual control and pUL37x1 over-expressing lines



S3. pUL37x1 over-expression demonstrated the protection of cell death against staurosporine when silencing with Scr siRNA compared with SH-SY5Y cells (control-1). However, there was no significant in the percentage of LDH release between pUL37x1 over-expressing lines with control SH-SY5Y cells when silencing with Bax siRNA. Data were presented as mean±S.E.M. Statistics were performed by one-way ANOVA with Dunnett's post-hoc analysis. (*, $p<0.05$, **, $p<0.01$, ***, $p<0.001$)

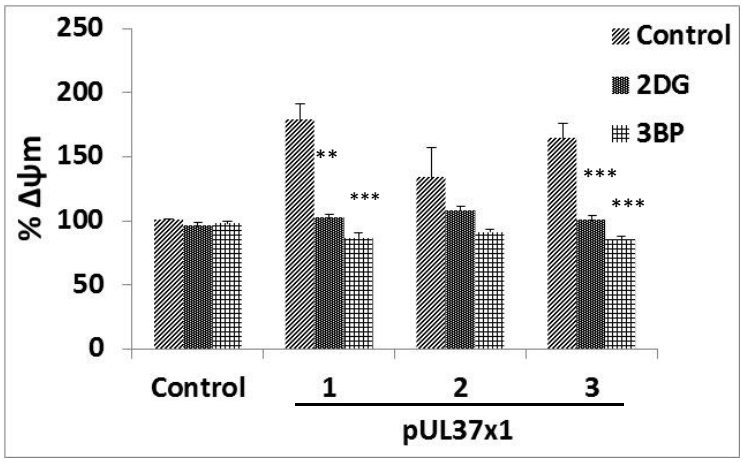
S4 The protection of pUL37x1 Over-expression Was Mitochondrial Hyperpolarization and Glycolysis-dependent: presented by individual control and pUL37x1 over-expressing line

S4-1



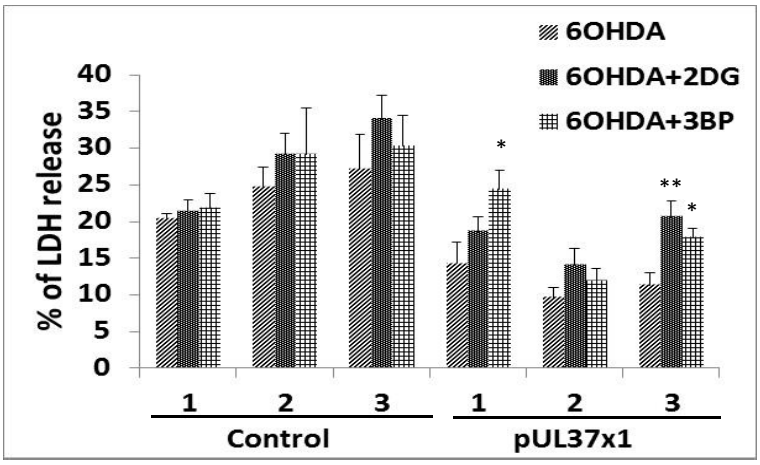
S4-1 pUL37x1 over-expression failed to protect cell death upon 1μM CCCP treatment for 48 hours. There was no significant difference in the percentage of LDH release between SH-SY5Y cells (control-1) with all pUL37x1 over-expressing lines. Data were presented as mean±S.E.M. Statistics were performed by one-way ANOVA with Dunnett's post-hoc analysis.

S4-2



S4-2. Glycolytic inhibitors reversed the hyperpolarization from pUL37x1 over-expression. pUL37x1-1 and 3 lines demonstrated a significant reduction of hyperpolarization upon either 10μM 2-deoxyglucose (2DG) or 5μM 3-Bromopyruvate (3BP) treatment. Data were presented as mean±S.E.M. Statistics were performed by one-way ANOVA with Dunnett's post-hoc analysis. . (**, p<0.01, ***, p<0.001)

S4-3



S4-3. Glycolytic inhibitors attenuated the cell death protection of pUL37x1 over-expression. There was no significant change of the percentage of LDH release when either 10μM 2-deoxyglucose (2DG) or 5μM 3-Bromopyruvate (3BP) co-applied with 6-OHDA in control cells. However, in pUL37x1 over-expressing lines, there was a significant increase of the percentage of LDH release at that condition. Data were presented as mean±S.E.M. Statistics were performed by one-way ANOVA with Dunnett's post-hoc analysis. . (*, $p<0.05$, **, $p<0.01$)